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(57) Abstract

The invention provides methods for changing the metabolic pathways of micro–organisms in the presence of a certain carbon source and uses of such changes, as well as micro–organisms and uses of such changes, as well as micro–organisms produced by these methods. In a preferred embodiment the invention provides new yeast strains with improved biomass yields, a process to obtain these yeasts and the potential application of these yeasts are provided. The biomass yield is improved by the introduction into a yeast of a DNA construct conferring altered expression of a gene encoding a protein controlling transcription of a number of glucose–repressed genes. The yeasts are less sensitive to glucose repression, resulting in an increase in respiratory capacity, reduction of ethanol production and increased conversion of sugar into biomass.

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METHODS FOR MODULATING METABOLIC PATHWAYS OF MICRO-ORGANISMS AND MICRO-ORGANISMS OBTAINABLE BY SAID METHODS

The present invention relates to the field of biotechnology, in particular to the field of culturing micro-organisms, in particular yeast.

Culturing of micro-organisms is a relatively old technique which is well established and well understood by persons skilled in this art. It usually involves bringing a micro-organism of interest into a culture medium wherein it can survive, grow and divide. The culture medium usually comprises all the necessary nutrients for the micro-organism to be able to do this.

Micro-organisms are cultured for many different purposes. These include the production of biomass, the production of antibiotics, the production of useful proteins expressed by micro-organisms (be it naturally or through genetic engineering), the production of micro-organisms useful themselves (for instance in brewing or baking bread, leavening of dough, etc.) Because of its relatively long history and its many uses the techniques for culturing micro-organisms have been very well optimised, so that further gains in yield or growth rate of the micro-organism to be cultured are difficult to achieve. However, because of the cost of culturing micro-organisms and the large amounts needed, such improvements (however small percentage-wise) remain very desirable.

One of the problems of culturing micro-organisms is that they often show preference for certain carbon sources, which carbon sources do not result in the best yields and/or growth rates of the micro-organism. Often the availability of such a preferred carbon source will lead to repression of the

metabolism of other available carbon sources (which other carbon sources often do result in higher yields).
For instance, *S. cerevisiae*, as many other micro-organisms, shows marked preferences for certain sources of carbon, nitrogen and energy. One such preference concerns the use of glucose above other fermentable and non-fermentable carbon compounds (see 1,2). This behaviour causes diauxic growth of this yeast when cultured on mixtures of carbon sources that include glucose. Yeast cells growing on glucose display high growth rates, presumably related to the ease with which intermediates can be derived from the catabolism of this sugar. Glucose has radical consequences for the enzyme complement and metabolic patterns in the yeast cell (Fig. 1). During growth on this sugar, enzymes required for metabolism of other carbon sources are either absent or strongly reduced in amount as a result of active degradation of mRNAs or proteins (catabolite inactivation), repressed synthesis of mRNAs (catabolite repression), or both. Such enzymes include permeases and key enzymes involved in the utilization of various sugars, enzymes of gluconeogenesis and the glyoxylate cycle. In addition, synthesis of components of the mitochondrial respiratory chain is repressed, resulting in a low respiratory capacity. Glucose-repressed cells, or cells pulsed briefly with excess glucose produce ethanol by decarboxylation of cytosolic pyruvate and subsequent action of alcohol dehydrogenase. This series of reactions, known as the Crabtree-effect, regenerates cytosolic NAD^+ required for glycolysis. Although the basis of the Crabtree response is largely unknown (3), the occurrence of these reactions during large scale production of *S. cerevisiae* is undesirable because it reduces cell yield.
Suppression of ethanol production by yeast cells growing on glucose is currently achieved by limitation of the supply rate of the sugar. This procedure is only partially successful in that incomplete mixing can trigger a short-term Crabtree response. Additionally it suffers from the drawback that cells are forced to grow below their maximum capacity.

These or similar problems occur in many micro-organisms, particularly in eukaryotes, more in particular in yeasts. The present invention provides a general mechanism by which such problems may be solved in that it provides a method for producing a micro-organism of which a metabolic pathway has been modulated, for instance a micro-organism of which the sugar/glucose metabolism has been shifted from aerobic fermentation towards oxidation, comprising providing said micro-organism with the capability of inhibition or circumvention of the repression of the oxidative metabolism of glucose induced by the availability for the micro-organism of said carbon source. Surprisingly we have found that by simply interfering at a single (well-chosen) spot in the complex regulatory mechanisms of metabolic routes in (in particular) eukaryotic micro-organisms, it is possible to redirect said metabolic routes from one mechanism (fermentation) to another (oxidation). Because of the complexity of the regulatory systems surrounding metabolism it is generally believed that interference at a single point would be unlikely to be of any significance (because of all kinds of positive and negative homeostatic mechanisms which would restore the original situation) or would be deleterious if not disastrous (if it were capable of disrupting the feedback-mechanisms). We have found that a simple modification, in particular at the level of transcription activation does lead to the desired switch in metabolic mechanism, without disrupting the metabolism of the micro-organism.

In particular, the invention solves this problem when the preferred carbon source of the micro-organism is glucose, which is the case for many micro-organisms, in particular yeasts. One of the most important yeasts in industry is (of course) *Saccharomyces cerevisiae*. For that reason we have chosen this micro-organism as a model for explaining our invention. Because of its importance it is of course also a highly preferred embodiment of the present invention. Other

well known industrial yeast strains such as Hansenula, Kluyveromyces and other strains are of course also within the scope of the present invention.

It will be understood that the result of the preference for glucose leads the metabolism down the aerobic fermentative pathway in many cases, as will be explained below. It will also be clear that for yield in biomass and/or production of useful proteins, etc. the oxidative/gluconeogenesis pathway is to be preferred. This pathway is often part of the metabolism that is repressed when glucose is available and which is used when other carbon sources are available. Thus an important aspect of the present invention is to provide a method according whereby the repressed metabolism is restored to a significant extent by activation of the pathways for metabolism for the non-preferred carbon sources.

It is preferred that said activation is achieved by providing the micro-organism with at least one transcriptional activator for at least one gene encoding an enzyme in said pathways.

A very suitable and preferred way of achieving said activation is one whereby the transcriptional activator is provided by introduction into the micro-organism of a recombinant nucleic acid encoding said activator. Said recombinant nucleic acid is preferably an expression vector. Such a vector may be an autonomously replicating vector, but it is preferred to use vectors that integrate in the host genome. However, it may also be achieved by other means, such as mutation (site directed).

There are two ways of having the transcriptional activator expressed. In one embodiment of the invention the transcriptional activator is constitutively expressed by said micro-organism. In an alternative embodiment the transcriptional activator can be expressed by the micro-

organism upon induction, for instance by the presence of glucose. The person skilled in the art will be able to determine which one is to be used for different circumstances and desired end results. In many instances it will be preferred that the vector used to introduce the activator is capable of integration into the genome of the host. In other embodiments a self-replicating vector may be used.

A very efficient way of achieving the derepression of the metabolic pathways other than those active in the presence of glucose is one whereby the transcriptional activator provided is a Hap4 protein or a functional equivalent, derivative or fragment thereof. A functional equivalent or derivative or fragment is defined as a molecule still having the same activity in transactivating the relevant genes from the relevant pathways (in kind, but not necessarily amount). Apart from being useful for biomass production and other uses of the micro-organisms themselves, another important use includes the production of recombinant proteins, homologous or heterologous. Thus the invention also provides methods for producing micro-organisms according to the invention, which micro-organisms further comprise a nucleic acid encoding such a protein of interest. Many proteins of interest have been disclosed and have been produced in yeast or other micro-organisms. They do not need to be reiterated here.

The micro-organisms produced by the methods according to the invention are also part of the invention. They are improved in many aspects, when compared with the organisms they are derived from, for instance they can have improved biomass yield upon culturing; they may show increased glucose oxidation; they may display increased oxidative sugar metabolism and/or reduced aerobic fermentation. Normally, under anaerobic culturing conditions the micro-organisms according to the invention will behave essentially the same as the corresponding micro-organism not provided with the modulation of this metabolic pathways.

The process to obtain the improved micro-organisms, in particular improved yeast has applications in all industrial processes in which optimal conversion of sugar into biomass is required. Use of these improved yeasts will lead to reduction of costs because of reduced process times and increased amounts of biomass per consumed glucose. This invention is well applicable in, for example, the (aerobic) production process of yeast for bakeries, or as source of flavour-enhancing yeast extracts. Furthermore this invention will lead to increased production of metabolites and heterologous gene products, such enzymes, precursors for chemicals, biosurfactants and fatty acids for application in pharmaceutical, agricultural or food sectors.

Concerning the application in baker's yeast or brewer's yeast, it should be noted that the reduction of alcohol formation in the production phase does not negatively affect growth or alcohol production during anaerobic fermentation, which is crucial for leavening of the dough or for the brewing process. This invention reduces alcohol production only when the yeast can make use of its enhanced respiratory capacity, i.e. under aerobic conditions.

The invention may also be applied with respect to manipulation of glucose repression or even glucose inactivation of processes not directly related to respiratory function of yeast. The uptake and metabolism of carbon sources other than glucose, such as galactose, sucrose or maltose is repressed by glucose. Yeast fermentation in lean dough depends on maltose as main substrate, which is produced in the dough from starch by action of amylases present in the flour. The flour contains in addition variable amounts of other sugars amongst which glucose. Maltose permease, responsible for the translocation of maltose across the plasma membrane and maltase, the maltose metabolising enzyme, are both subject to glucose repression and inactivation (4).

Since this negatively affects CO_2 production and thereby leavening activity, tools to reduce the glucose effect on maltose uptake and utilisation would be useful. Therefore it is noteworthy this invention will even improve leavening activity of said yeast strains.

Detailed description

As stated before, a very important micro-organism is *Saccharomyces cerevisiae*. We will explain in detail how its reaction to the presence of glucose may be changed, as exemplary for other micro-organisms. In the presence of glucose Saccharomyces shows a "Crabtree" response and switches to ethanol production through aerobic fermentation.

The present invention in one embodiment offers a solution to such problems by the construction of production strains of micro-organisms in particular yeasts, more specifically *S. cerevisiae* in which the Crabtree-effect is reduced or absent. The principle of the approach is the controlled de-regulation of glucose-repressible genes by overexpression of a specific transcriptional activator from a promoter insensitive to glucose control. The resultant shift in balance from fermentative to oxidative metabolism leads to increased growth rates and reduced ethanol production.

Glucose control of metabolism in *S. cerevisiae*

The extensive changes in enzyme complement during a shift from oxidative/gluconeogenic to fermentative growth are, in the vast majority of cases, the result of induction or repression of transcription of the corresponding genes in response to glucose. Genes whose expression is repressed by this sugar can be divided into three groups:

1. Genes required for the uptake and metabolism of other carbon sources, such as galactose, sucrose, maltose, glycerol, lactate and ethanol.
2. Genes unique to gluconeogenesis and the glyoxylate cycle.

3. Genes coding for Krebs cycle enzymes and components of the respiratory chain.

Although each group displays distinct features of regulation, a number of common transcription factors and mechanisms are involved and these form the main glucose repression/deprepression pathway (MGRP; 2). As shown in Fig. 3, key events in this pathway are the activation or inhibition of a number of key transcription factors in response to a signal generated by glucose. The nature of this signal is unkown. Its main effect is, however, to inhibit or counteract the action of the Snf1/Snf4 complex, a protein serine/threonine kinase, which is thought to alleviate transcriptional repression and promote transcriptional activation at glucose-regulated promoters (5). Although as yet, there is no evidence that Snf1/Snf4 directly phosphorylates a transcriptional regulator, genetic studies suggest that important direct or indirect targets for Snf1/Snf4 are the transcription factor Mig1, Ssn6/Tup1 and Hap2/3/4. Mig1 is a zinc-finger protein which acts as a transcriptional repressor at many glucose-repressible genes (6) Ssn6/Tup1 acts as a repressor of transcription at a large number of genes, probably in combination with gene- or family-specific transcription factors (7). The Hap2/3/4 complex is, in contrast, an activator of transcription. It is required for induction of transcription by non-fermentable carbon sources of a limited number of genes encoding proteins involved mainly in mitochondrial electron transport, Krebs cycle, haem biosynthesis and gluconeogenesis (8, 9, 10, 11, 12). Transcriptional regulation by the Hap2/3/4 complex is the main mechanism for coordinating the derepression of these enzymes in response to changes in carbon status of the medium (11, 13). The activity of the Hap complex is controlled by the availability of the activator subunit Hap4, whose synthesis is approximately 5-fold repressed by glucose (13).

Transcription and transcriptional control in yeast

A typical yeast promoter consists of several cis-acting elements that function as target sites for regulatory proteins (Figure 2). The position of transcription initiation by the RNA polymerase II complex is located at the initiation site (I). The TATA-box (T) has been shown to be essential in many promoters for transcription initiation to occur. It is the target site for the basal RNA polymerase II transcription factor TFIID, which nucleates the assembly of the other basal transcription factors and RNA polymerase II into a stable preinitiation complex. In addition to these basal control elements, at least one upstream activation site (UAS) is required for transcription. UAS elements function as DNA-binding sites for transcriptional regulatory proteins, that are thought to interact with the basal transcriptional machinery to mediate specific regulation. In many instances, yeast promoters consist of several TATA- and UAS-elements, which together determine the rate of transcription of the adjoining gene. In addition, yeast promoters may contain operators or upstream repressor sites (URS) and upstream induction sites (UIS). By binding of specific proteins these elements contribute to the overall transcriptional regulation.

Carbon source-dependent transcriptional regulation by the Hap2/3/4 complex

Carbon source dependent transcription of genes encoding a number of components of the mitochondrial respiratory chain and enzymes of gluconeogenesis is regulated by the Hap2/3/4 complex. Hap2 and Hap3 were first identified as proteins capable of binding to the UAS2 region of the gene encoding iso-1-cytochrome c in S. cerevisiae (14). This region, responsible for carbon source response, contains a sequence motif closely resembling the CCAAT box element found in many other eukaryotic promoters. The two proteins bind to DNA in an interdependent manner. Hap4 appears not to contact DNA directly, but is necessary for DNA binding of the other two

proteins. Sequence analysis reveals a C-terminally located, highly acidic region whose presence is necessary for activity. Replacement of this region by the activation domain of the yeast Gal4 protein restores activity, suggesting that it provides the principal activation domain of the DNA-bound Hap2/Hap3 complex (13). Transcription of the genes for Hap2 and Hap3 in *S. cerevisiae* is not substantially affected by carbon source, but expression of the gene for Hap4 is glucose repressible (13). This suggests that Hap4 is the key component of the complex in terms of its ability to regulate transcriptional activity in response to carbon source.

Insight into the structure of the Hap2 and Hap3 proteins has been obtained by the isolation and sequencing of the corresponding genes. The *HAP2* gene encodes a 265 residue protein, of which an evolutionarily-conserved 65 amino acid core in the highly basic C-terminal region is necessary and sufficient for both complex formation and binding to DNA (Fig. 4). The *HAP3* gene encodes a 144 residue protein, which contains a 90 amino acid core (B-domain) required for complex formation and DNA-binding (Fig. 5). The *HAP4* gene encodes a 554 residue protein containing two highly acidic regions in its C-terminal domain (13; Fig. 6). Both of these appear to be necessary for transcriptional activation.

Genes corresponding to *HAP2* and *HAP3* have been isolated from a wide range of organisms (15). The encoded proteins form a heteromeric complex called NF-Y, CBF or CP, which activates transcription by binding to the evolutionarily conserved CCAAT box element. Hap2- and Hap3-homologous subunits make similar contacts with DNA. For the human CP1 complex, it has been shown that the Hap2 and Hap3 homologues are exchangeable in vitro with those of yeast (16). However, although the human CP1 complex consists of more than two subunits, none of these appear to correspond to the *S. cerevisiae* Hap4 protein. The Hap homologous complexes are not specifically involved in induction of genes under certain growth conditions, but

function as general transcription factors required for basal level expression of a large number of genes.

As the Hap complex (in the yeast *Saccharomyces cerevisiae*) is involved in the regulation of many metabolic processes, it is to be expected that modification of its expression has a profound effect on the cell's physiology. With regard to anabolic processes, to date not much is known about its role but with regard to the catabolic network (that is, the energy conserving machinery) in *S.cerevisiae*, synthesis of components of the respiratory chain (hence mitochondrial) is to a significant extent under control of the Hap2/3/4 complex. It seems justified to conclude that Hap-dependent regulation is at least involved in the physiological phenomenon known as the Crabtree effect. Its direct physiological impact is a catabolic shift from respiratory to fermentative catabolism whenever elevated levels of glucose are present. This results in a significant decrease in the efficiency of energy conserved: only 2 moles of ATP are synthesized per glucose fermented to ethanol and carbon dioxide, whereas the number of moles of ATP (equivalents) per glucose oxidized (to carbon dioxide) is manyfold higher (the exact number still being a matter of debate). An important indirect effect is a decrease in anabolic capacity: it is known that the maximal obtainable growth rate of *S. cerevisiae* is highest under conditions that allow respiration. As a consequence, whenever conditions are such that the catabolic flux into respiration is increased, the yield value ($Y_{glucose}$, defined as the amount of cells obtained per glucose consumed) will be considerably higher and the organisms will grow faster. In other words, under such conditions the partitioning of the total carbon flux over the catabolic and the anabolic flux will be directed towards the latter. Thus, a larger part of the carbon source is directed towards biomass formation and a given biomass concentration is achieved in a reduced time span. Due to the said regulation (Crabtree effect), fully respiratory catabolism

can only occur under aerobic conditions with continuously very low glucose availability, a condition not often met in practical settings.

According to the invention, the inhibition or circumvention of glucose-regulated partitioning of the catabolic fluxes should result both in increased $Y_{glucose}$ values and higher growth rates. Here, it is important to note that it can be foreseen that a relatively small increase in the respiratory flux may result in a significant gain in biomass yield due to the large difference in the energetic efficiency between respiration and fermentation. All anabolic processes will be enhanced and since anabolism comprises protein synthesis it is to be expected that said increase is beneficial not just to biomass production per se only but also to the production of specific proteins.

Modification of the expression of glucose repressed genes can be achieved by interference in other factors of the glucose repression signalling cascade besides Hap4. All mutations in upstream regulators of the cascade (see Figure 3), like Snf1 or Hxk2, do alleviate glucose repression of SUC2, GAL, MAL and respiratory genes, but these mutants display a wide range of phenotypic defects (1, 17, 18) and are not suitable for industrial application of yeast. De-repression of SUC, MAL and GAL genes can also be accomplished by removal of the general glucose repressor Mig1, which results in partial alleviation of glucose control of maltose metabolism in a laboratory strain (19) This does however not affect glucose repression of respiratory genes, and no change in fermentative-oxidative metabolism, growth rate or cell mass yield.

We have now found that yeast, transformed with a construct that overexpresses the *HAP4* gene, becomes insensitive to glucose repression of transcription of a number of genes, amongst which genes encoding respiratory components. This

results in increased respiratory capacity of this yeast strain and reduced ethanol production. This altered aerobic sugar metabolism leads to a drastic increase in biomass yield and a significantly increased growth rate.

The present invention provides a transformed yeast with an reduced aerobic fermentation rate of glucose which comprises the introduction into yeast of a DNA construct which contains an homologous gene encoding a protein de-regulating glucose repression of a number of genes in the said yeast.

The homologous gene in this invention, *HAP4* is cloned into a circular vector DNA construct which is transformed into yeast by a procedure described in the following section. The vector contains DNA sequences that enables replication in both E. coli and yeast, sequences that enable cloning of DNA fragments into the vector, a yeast marker gene and a bacterial marker gene that enables selective maintenance in yeast or *E. coli* respectively. After transformation of the vector into yeast, it will be self-replicating and be maintained as long as selective pressure against plasmid loss is sustained. During non-selective propagation (i.e. growth in the presence of leucine in this particular case), the plasmid will be lost. From a practical point of view, it is preferable to grow yeast non-selectively. This will entail alteration of expression of *HAP4* by integration of the altered gene in the yeast chromosome. By "altered" is meant the exchange of the natural promoter by another promoter which is constitutively active, or by integration of a DNA construct consisting of such a promoter fused to the *HAP4* gene on a different locus on the chromosome, e.g. the SIT2 locus (20). Integration of homologous yeast sequences is a well described and efficient technique (21) and can be easily applied to (industrial) yeast strains. The yeasts thus obtained are stable transformants and the altered *HAP4* gene can be maintained in the genome without selective pressure.

Yeast with a chromosomally altered *HAP4* gene can be constructed in such a way that the transformants are completely devoid of prokaryotic DNA, in contrast to the transformants also described in this invention which contain a plasmid harbouring prokaryotic DNA sequences. By integration of only homologous DNA, originating from Saccharomyces, one can improve the yeast without introducing any heterologous DNA.

The present invention comprises induction of changes in glucose metabolism by overexpression of a key transcriptional regulator of oxidative metabolism of yeast by introducing in the yeast a DNA construct expressing HAP4 under control of the ADH1 promoter, whose activity is enhanced approximately ten-fold by glucose. The elevated expression level has alternatively been achieved by using other strong promoters, which are constitutively expressed, independently of the presence of glucose. This is of particular importance for application in fed-batch cultures, where the glucose concentration is kept low in order to minimise the Crabtree-effect. Several promoters belonging to genes encoding enzymes of the glycolytic cycle, like glyceraldehyde-phoshate dehydrogenase (GPD) or genes involved in ribosomal expression, such as promoters for the transcription of elongation factors (EF) are well characterised and widely used for overexpression of yeast genes. These promoters have been isolated from *S. cerevisae* and cloned in expression vectors for yeast (22). The coding region of the isolated HAP4 gene was cloned behind these promoters, after which the promoter-HAP4 fusions were recloned in such a construct that the HAP4 gene with altered regulation of expression could be integrated into the yeast genome. This procedure leads to stable transformants which exhibit all the advantages described above due to more oxidative growth and which in addition do not contain any prokaryotic DNA sequences.

The yeast strains described in this invention comprise both laboratory strains and industrial production yeast strains. The overexpression of Hap4 was first achieved in a laboratory strain by means of introduction of a self-replicating plasmid harbouring the *HAP4* gene under control of the *ADH1* promoter. The metabolic behaviour of this engineered strain was tested in detail as described below, showing a significant increase in oxidative metabolism when grown on glucose. Industrial production strains were transformed with the same plasmid, which was slightly modified by introduction of a dominant marker thereby enabling selection of transformants containing the plasmid overproducing Hap4. The response of the industrial strains to Hap4 overexpression was similar to that of the laboratory strain, i.e. glucose repression of respiratory function is alleviated and therefore glucose metabolism has shifted from fermentative towards oxidative metabolism. To obtain stable yeast strains with all the advantages described above, genomic integration of constitutively expressed Hap4 was carried out, as described in detail in the following sections.

Experimental

Saccharomyces cerevisiae strain DL1 has been transformed with YCplac111::ADH1 (without any gene placed behind the ADH promoter) or YCplac111::ADH1-HAP4 (expression of HAP4 under control of ADH1 promoter). Transformation of the yeast strain with YCplac111::ADH1, the so-called "empty" plasmid is necessary to prevent any differences in physiology of the Hap4 overproducer and the wild type due to differences in e.g the plasmid encoded *LEU2* marker gene. The nomenclature of the transformed yeast strains is as follows: DL1 denotes strain Dl1 transformed with YCplac111::ADH1, whereas DL1HAP denotes DL1 transformed with YCplac111::ADH1-HAP4. The expression level of *HAP4* mRNA in these strains is depicted in Fig 9. Expression of *HAP4* in Dl1 is strongly repressed by glucose. Introduction of the plasmid with HAP4 under control of the ADH1 promoter leads to an increased expression level of *HAP4*

in DL1HAP which is grown on media containing 2% glucose. This level is comparable to the expression level of *HAP4* in wildtype DL1 cells when grown on non-fermentable carbon-sources, which do not repress transcription of *HAP4* and genes encoding respiratory components.

To study the effect of *HAP4* overexpression on transcriptional control of respiratory function, we first studied the mRNA levels of different genes encoding components of the respiratory chain. As shown in Figure 9, the elevated level of HAP4 in glucose containing medium leads to de-repression of transcription of *QCR8*, the gene encoding the 11kDa subunit of the yeast ubiquinol-cytochrome *c* oxidoreductase *(QCR)* complex of the respiratory chain. Comparable results were obtained for a number of other genes encoding respiratory components (Table I). Transcription of *SUC2*, a glucose repressed gene without an Hap binding box in the promoter region is not induced by overexpression of *HAP4* on glucose.

To test whether the increased level of mRNAs of respiratory components results in an higher respiratory capacity of the Hap4 overproducing strain, we measured oxygen consumption rates of cells as described in a previously section. Respiratory capacity of DL1HAP cells grown in shake flask cultures on complex media containing glucose is increased two-fold compared to wildtype cells (Table II). When grown in the presence of the non-fermentable carbon-source lactate, the respiratory capacity is further increased approximately five-fold to a level similar for both wildtype and Hap overproducing strains.

Further characterization of the physiological properties of the Hap4 overproducing strain required growth under controlled conditions (constant pH, aeration, stirring) in well defined mineral media (see section 'batch cultivation in fermentors'). Therefore, aerobic growth of Dl1 in a defined mineral salts medium containing 30 g/l (3%) glucose was

compared with growth of Dl1HAP cells. As calculated from the growth curves (not shown), DL1 grew exponentially with a specific growth rate of 0.16± 0.01h^{-1}, whereas the growth rate of DL1HAP was 0.18± 0.01h^{-1}. Overproduction of Hap4 thus results in an increased growth rate of 11%.

During a period of six hours during exponential growth, samples were taken with an interval of one hour to measure substrate consumption and biomass and product formation. The mean biomass yield during this time course, i.e. the amount of biomass formed per gram consumed glucose is 10.1 gram for DL1 and 14.8 gram for DL1HAP. Overproduction of Hap4 thus leads to 46% increase in biomass yield compared to wild type, which is rather constant during the experiment (see Figure 10).

Analysis of other carbon compounds present in the culture medium showed that ethanol production is significantly decreased (38%) in DL1HAP compared to the wild type (Table IV). The reduction in ethanol formation is accompanied by a 2.3-fold increase of the amount of acetate, whereas the amount of glycerol decreased 3.5-fold in DL1HAP cells. The oxygen consumption during the experiment was appoximately twice as high in DL1HAP compared to DL1. All data are thus consistent with a shift of carbon metabolism from fermentative towards oxidative metabolism due to overexpression of *HAP4*. This is further illustrated in Table V, which summarizes carbon fluxes in both strains. The amount of CO_2 produced via TCA cycle was calculated by the amount of oxygen consumed during the experiment.

When grown under anaerobic conditions, both strains are identical with respect to growth rate, ethanol production and biomass yield (data not shown). This implies that overexpression of *HAP4* only exhibits its effect during aerobic growth of yeast. Processes depending on anaerobic alcoholic fermentation, like brewing or dough leavening will

be unaffected by *HAP4* overexpression. This invention is therefore very well applicable in optimization of biomass yields in the aerobic production phase of industrial yeast strains.

Taking into account strain-dependent variations in glucose repression (23, 19), it should be mentioned that the regulation of expression of *HAP4* in several industrial strains was found to be similar to laboratory strains described above and in literature (13), i.e. the expression is repressed by glucose (data not shown). To obtain constitutive high expression levels of *HAP4* in the industrial strains, we integrated a DNA construct consisting of a constitutively active promoter (of glyceraldehyde-phosphate dehydrogenase, GPD1 and elongation factor 1-alpha, TEF2) fused to the coding region of the *HAP4* gene in the genome of the yeast strain. The integration plasmid is constructed in such a way that the promoter-HAP4 fusion is flanked by DNA sequences representing parts of the yeast SIT2 gene (see Fig 11 and 13). Integration of the total plasmid including the promoter-HAP4 fusion can therefore take place at the SIT2 locus in the yeast genome. The presence of a gene encoding acetamidase (amdS) enables cells which have integrated the plasmid to grow on medium containing acetamide as a nitrogen source, in contrast to untransformed cells which are unable to grow on this medium. Since the plasmid does not contain any sequences which enable replication in yeast, integration of the plasmid in the genome is required to acquire the ability to grow on acetamide.

Strains DS28911, DS18332 and DS19806 were transformed with the plasmid pKSPO2-GPDHAP4 or pKSPO2-TEFHAP4. Transformants with an integrated plasmid in the genome were selected on plates containing acetamide. Transformants containing pKSPO2-GPDHAP4 were named DS28911GH, DS18332GH,or DS19806GH, transformants containing pKSPO2-TEFHAP4 DS28911TH, DS18332TH

or DS19806TH. In a number of transformants the position of integration was analysed by Southern blotting, in which genomic DNA fragments containing SIT2 and/or HAP4 sequences were visualized (see Fig 13). The length of the different fragments revealed that integration preferentially took place at the *HAP4* locus, as shown in Figure 13 and 14. These (anaploïd) transformants contain both one or more glucose repressed *HAP4* genes and a HAP4 gene which is under control of a constitutively active promoter.

To test the expression level of HAP4 in these transformants, mRNA was analysed of cells grown aerobically in media containing 2% glucose. The mRNA expression level of one selected transformant of every strain containing either the GPDHAP4 (GH) or the TEFHAP4 (TH) is shown in Figure 15. Different levels of *HAP4* mRNA were obtained in the different strains and with the two different promoters. The effect of overexpression of HAP4 on expression of *QCR8*, a glucose-repressed gene encoding a subunit of the respiratory chain was analysed in four independent transformants. Strains exhibiting clear and reproducible de-repression of *QCR8* were selected for further studies and are shown in Figure 15. As a loading control a constitutively expressed gene (PDA, encoding a subunit of pyruvate dehydrogenase, (24)) was visualized, showing that the higher expression of *QCR8* is due to HAP4 overexpression. The integrated constitutively expressed HAP4 thus appears to result in alleviation of transcriptional repression of a respiratory component, analogous to the effect of the plasmid encoded HAP4 under control of the ADH1 promoter as described above.

To test the respiratory capacity of DS28911, DS18332 and DS19806 with the integrated GPDHAP4 (GH) or the TEFHAP4 (TH) fusion, we measured oxygen consumption rates of cells grown in medium containing 4% glucose. As shown in Table VI, overexpression of HAP4 on glucose results in an increase of the respiratory capacity, ranging from 2.2 to 4.1 fold the

value found in the corresponding wild type cells. The respiratory capacity of cells grown in lactate containing medium is 7.7 fold higher than in wild type cells. The allevation of glucose repression of respiratory function is thus only partial, but similar or even higher than obtained with the laboratory strain DL1 containing the ADH1-HAP4 fusion on plasmid. It can therefore be expected that the physiological advantages of the latter strain are also applicable to the industrial strains harbouring the integrated constitutively expressed HAP4.

Application of the modified industrial strains requires removal of any sequences that are not derived from yeast. The transformants contain the total plasmid pKSPO2-GPDHAP4 or pKSPO2-TEFHAP4 in the genome. The 'non-yeast' plasmid sequences can however be removed by homologous recombination of identical DNA segments in the genome, as depicted in Figure 13. Cells which are devoid of plasmid sequences can be selected by growth on medium containing fluoro-acetamide, which is toxic for cells still containing the gene encoding acetamidase. When the plasmid is integrated at the HAP4 locus, this recombination event will cause loss of the GPD- or TEF-HAP fusion as well, resulting in a wild type strain. Only after integration at the SIT2 locus the chromosomal arrangement is such that recombination can also result in cells that still contain the GPD- or TEF-HAP4 fusion. These 'clean' transformants now only contain yeast sequences and are suitable for industrial application.

To obtain these clean transformants, strains DS28911; DS18332 and DS19806 were transformed with plasmid pKSPO2-GPDHAP4 or pKSPO2-TEFHAP4 which was linearized at the *SfiI* site (see Figure 11B). This led to a higher efficiency of integration at the SIT2 locus, as shown by Southern blot analysis. Comparison of expected (Figure 13) and obtained (Figure 16A) DNA fragments of a number of transformants revealed a new set of strains overexpressing *HAP4* from the SIT2 locus. The expression levels of *HAP4*, *QCR8* and *PDA1* were determined by

northern blotting of mRNAs isolated from cells grown in medium containing 2% glucose. As shown in Figure 17A, also in these strains the elevated expression of *HAP4* resulted in alleviation of glucose repression of *QCR8*. Subsequently we selected fluoro-acetamide resistant cells that had lost their plasmid sequences (including the acetamidase gene) by homologous recombination. Transformants which retained the GPD- or TEF-HAP4 fusion in the genome were selected by verification of the correct chromosomal arrangement by Southern blot analysis. Figure 16B shows three examples of clean transformants derived from transformants DS18332GH15 and DS18332TH25. As shown in Figure 17B, removal of the plasmid sequences has no negative effect on either HAP4 overexpression or QCR8 derepression. The effect of the HAP4 overexpression in these strains on oxidative metabolism can therefore be expected to be similar to those described above.

EXPERIMENTAL PROCEDURES

Cloning techniques

All general cloning techniques (plasmid isolation, restriction, gel electrophoresis, ligation) were carried out as described by Maniatis et al., Molecular Cloning, A Laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1982). DNA restriction enzymes were purchased from New England Biolabs (Biolabs), Boehringer Mannheim (Boehringer) or Bethesda Reseach laboratories (BRL). These enzymes were used in conditions and buffers described by the manufacturer.

Construction of recombinant plasmid YCplac111::ADH1-HAP4

The centromeric plasmids YCplac111::ADH1 and YCplac111::ADH1-HAP4 are capable of self-replicating in E. coli and in yeast and contains the ADH1 promoter region without (YCplac111::ADH1) or with the coding sequence of HAP4. The construction of YCplac111::ADH1-HAP4 is outlined in Figure 7.

YCplac111::ADH1 is derived from YCplac111 (25) and contains between the BamHI and the SmaI site a 723 bp EcoRV promoter fragment from pBPH1 (26). Vector pBPH1 was derived from pAC1 (27), which is a YCp50 derivative which carries the BamHI/HindIII fragment from pMAC561 containing the yeast alcohol dehydrogenase I promoter (28).

The coding region of *HAP4* was cloned behind the ADH1 promoter in the former construct by isolation of *HAP4* from pSLF406 (13). pSLF406 was digested with BspHI, which cleaves HAP4 at position -1 relative to the start codon the coding sequence. The BspHI end was blunted and subsequently the *HAP4* fragment obtained by cleavage of the DNA with PstI and isolation from agarose gel. YCPlac111 was cleaved with SmaI and PstI and ligated with the *HAP4* fragment to generate YCplac111::ADH1-HAP4.

Construction of recombinant integration plasmids p425-GPDHAP4, p425-TEFHAP4, pKSPO2-GPDHAP4 and pKSPO2-TEFHAP4

The plasmid pKSPO2 is used for integration of a fusion of HAP4 and the GPD1 or TEF2 promoter. These fusions were first constructed in shuttle vectors containing either the GPD1 (p425GPD) or the TEF2 promoter region (p425TEF) (22). A 2621 bp fragment from pSLF406 (13), containing the HAP4 gene, was isolated after digestion with BspHI, blunting and digestion with PstI as described in the previous paragraph. This HAP4 fragment was cloned in SmaI and PstI sites of the vectors 425GPD or p425TEF, resulting in the vectors p425-GPDHAP4 and p425-TEFHAP4 respectively. These shuttle vectors can self-replicate in E. coli and in yeast, but will only be maintained in yeast when selective pressure is present, i.e. when the recipient strain is LEU2 auxotrophic. In industrial strains without auxotrophic markers, integration of the GPDHAP or TEFHAP fusion is required to maintain the altered HAP gene stable in yeast. The promoter-HAP4 fragments were therefore recloned into the integration vector pKSPO2 (constructed at and provided by Gistbrocades). A 3359 bp Ecl136II-SalI GPDHAP4 fragment was isolated from p425-GPDHAP4

and a 3081 bp Ecl136II-SalI fragment was isolated from p425-TEFHAP4, which were cloned into pKSPO2 digested with SmaI and SalI. The different cloning steps are schematically drawn in Figure 11. Figure 12 shows the DNA sequence of the GPDHAP4 and TEFHAP4 fusions.

Transformation procedures

Transformation of *E.coli* was carried out using the electroporation technique , using a Biorad E.Coli pulser according to the description of the manufacturer.

Yeast cells were transformed according to the LiAc method described by Ito et al (29). Transformants of strain DL1 with the plasmid YCplac111::ADH1-HAP4 were selected on plates containing 2% glucose, 2% agar, 0.67% Yeast Nitrogen Base (Difco) supplied with the required aminoacids but lacking leucine. The industrial strains DS28911, DS18332 and DS19806 were plated on medium consisting of 1.8% nitrogen-free agar (Oxoid), 1.17% Yeast Carbon Base (Difco), 30 mM phosphte buffer pH 6.8 and 5mM acetamide (Sigma).

Counterselection of transformants

To select for transformants which after integration of the plasmid pKSPO2-GPDHAP4 or pKSPO2-TEFHAP4 recombine the plasmid sequences out of the genome (see Figure 13), counterselection was carried out on plates containing 1.8% nitrogen-free agar, 1.17% Yeast Carbon Base, 30 mM phosphate buffer, 60 mM fluoro-acetamide (Fluka) and 0.1% $(NH_4)_2SO_4$. Transformants were grown in YPD medium for 60 to 70 generations by daily dilution of the cultures for 3 to 4 days. Aliqouts of the cultures were plated on the fluoro-acetamide containing plates, which selects for cells which have recombined out the acetamidase gene. The presence of the GPDHAP4 or TEFHAP4 fusion was tested by Southern blot analysis.

Growth of yeast in flask-batch cultures

For shake flask cultivation, yeast cells were grown in either YPD (1% Yeast Extract, 1% BactoPeptone, 3% D-Glucose), YPEG (Yeast Extract , 1% BactoPeptone, 2% Ethanol, 2% Glycerol), YPL (lactate medium: 1.5% lactic acid, 2% Na-lactate, 0.1% Glucose, 8mM $MgSO_4$, 45 mM $(NH)_2HPO_4$, 0.5% Yeast-extract) or in mineral medium (0.67% Yeast Nitrogen Base) containing 3% D-glucose and supplemented with the appropiate aminoacids to obtain selective pressure for maintenaince of the transformed plasmid.

All cultures were inoculated from precultures which were prepared by inoculation of 5 ml medium with a colony from a plate. For northern analysis and oxygen consumption capacity measurements, the wild type and modified industrial yeast strains were precultured overnight in YPD and strain DL1 containing YCplac111::ADH1-HAP4 and Dl1 containing the empty YCplac111 vector were precultured overnight in mineral medium with additional amino acids. These cultures were used to inoculate 100ml YPEG and/or YPD medium and grown overnight at 28°C to OD_{600} 1.5, after which cells were harvested by centrifugation.

Growth of yeast in fermentor-batch cultures

Transformed yeast cells were grown in selective mineral Evans medium containing 30 g l^{-1} D-Glucose and supplemented with 40 mg l^{-1} uracil and L-histidine. The mineral medium contained : $NaH_2PO_4.2H_2O$, 10mM; KCl, 10 mM; $MgCl_2.6H_2O$, 1.25 mM; NH_4Cl, 0.1 mM; Na_2SO_4, 2 mM; $C_6H_9NO_6$, 2 mM; $CaCl_2$, 20 mM; ZnO, 25.3 mM; $FeCl_3.H_2O$, 99.9 mM; $MnCl_2$, 50.5 mM; $CuCl_2$, 5 mM; $CoCl_2$, 10 mM; H_3BO_3, 5.2 mM; $Na_2MoO_4.2H_2O$, 0.08 mM. After heat sterilization of the medium at 120°C, filter sterilized vitamins were added to final concentrations per liter of : myoinositol, 0.55 mM; nicotineacid, 0.16 mM; Ca-D(+)panthothenate, 0.02 mM; pyridoxine-HCL, 0.013 mM; thiamine-HCl, 0.006 mM; biotin, 0.02 mM.

Cultivation was performed at 28°C in New Brunswick Scientific Bioflow fermentors, at a stirrer speed of 900 rpm. The pH was

kept constant at pH5.0 via the automatic addition of 2 mol l^{-1} NaOH. Antifoam (BDH) was added automatically at fixed time intervals. The fermentors were flushed with air at a flow rate of 0.8 l min^{-1}. The starting working volume of the cultures was 1.0 or 1.4 liter. Samples of 30 ml were taken every hour for analysis of culture purity, dry weight, substrate consumption and product formation.

Determination of culture optical density and dry weight

Optical cell density of cultures was measured in a spectrophotometer at 600nm. The dry weight of cultures was determined by centrifugation of 10.0 ml of culture, washing cells with demineralized H_2O, and drying the cellpellet overnight at 80°C. Parallel samples varied by less than 1%.

Substrate consumption and product formation in liquid medium

Concentrations of carbon compounds, like glucose, ethanol, glycerol, acetate and pyruvate were determined by HPLC analysis using an Aminex HPX87H organic acids column of Biorad at 65°C. The column was eluted with 5 mM H_2SO_4. Detection was by means of a 2142 refractive index detector (LKB) and SP4270 integrator of SpectraPhysics.

Analysis of CO_2 production and O_2 consumption

During cultivation in fermentors, the dissolved carbondioxide concentration was continuously monitored by a cervomex IR PA404 gas analyzer and oxygen by a Taylor cervomex OA 272 gas analyzer. The absolute amounts of gas consumption/production during the time course of the experiment was calculated by the mean of the gas concentration, corrected for the decreasing volume of the culture.

For oxygen consumption capacity measurements of flask-batch grown cells, the cells were harvested, washed three times with ice-cold demineralized H_2O, and resuspended in oxygraph buffer (1% Yeast Extract, 0.1 % KH_2PO_4, 0.12 % $(NH4)_2SO_4$, pH 4.5) at 200 OD units ml^{-1}. Oxygen consumption capacity of the

cells was measured with a Clark electrode, with 0.1 mM ethanol as substrate.

RNA isolation, Northern analysis and labelling of DNA fragments

Cells were harvested and RNA was isolated, separated on a non-denaturing 1.2% agarose gel and transferred to a nitrocellulose filter as described by De Winde (30). Prehybridization was performed in hybridization buffer containing 50 microgram/ml single stranded salm sperm DNA (50% formamide, 25 mM NaPi pH 6.5, 5xSSc, 5xDenhardt, as described by Maniatis (31). DNA fragments used as probes in this study include a 840 bp HindIII-SalI fragment from pJH1 (30); a 1.6 kb BamHI-KpnI fragment containing the yeast actin gene (24); ; a 2.5 kb HindIII-SalI fragment from YE23SH containing the QCR2 gene (25); a 1333 bp NcoI-HindIII fragment from pAZ6 containing the yeast PDA1 gene (24) and a 1.2 kb BamHI-HindIII fragment from YE23R-SOD/SUC containing the SUC2 gene (26). Fragments were labeled ^{32}P by nicktranslation as described by Maniatis et al. Labeled probes were added to the prehybridization buffer and hybridization was performed overnight at 42°C. Blots were washed once with 2x SSC 0.1%SDS, twice with 1xSSC, 0.1%SDS and finally with 0.5x SSC, 0.1%SDS. Blots were air-dried completely and autoradiography was performed with Kodak Xomat 100 film or analysed by a Storm 840 Molecular Dynamics Phosphorimager.

Chromosomal DNA isolation and Southern blotting

Chromosomal DNA was isolated according to the method described by Hoffman and Winston (34). 10 microliter of the chromosomal DNA was digested with either BamHI or EcoRI. The digested DNA was separated on a 1% agarose gel and transferred to nitrocellulose filter as described in Maniatis (31) Prehybridization of the filters was performed at 65°C in 6xSSC, 5xDenhardt, 0.5% SDS and 100 microgram/ml salmon sperm DNA. After 4 hours prehybridization, a radioactive labeled

KpnI-XbaI fragment from pKSPO2-GPDHAP (see Figure 11B and 13) was added and hybridization was continued overnight at 65°C. Blots were washed once with 2x SSC 0.1%SDS, twice with 1xSSC, 0.1%SDS and finally with 0.5x SSC, 0.1%SDS. Blots were air-dried completely and radioactivity was visualised and analysed by a Storm 840 Molecular Dynamics Phosphorimager.

Description to the figures

Figure 1

Simplified view of the carbon metabolism in glucose-repressed *Saccharomyces cerevisiae* cells. Only a number of intermediates are shown, and specific pathways for the utilization of other carbon sources than glucose are only shown for maltose and galactose.

Figure 2

Transcriptional regulation of a typical yeast promoter. ATG denotes the start codon of the corresponding translational open reading frame. Abbreviations: UIS, upstream induction site; UAS, upstream activation site; URS, upstream repressor site; T, TATA-box, I, initiation site.

Figure 3

Schematic representation of the regulatory pathways involved in glucose repression of a number of genes in yeast. Activating functions are denoted as (+), repressing/inactivating functions as (-), and interactions which are not resolved yet are denoted as (?).

Figure 4

nucleotide and amino-acid sequence of the *HAP2* gene

Figure 5

nucleotide and amino-acid sequence of the *HAP3* gene

Figure 6
nucleotide and amino-acid sequence of the *HAP4* gene

Figure 7
illustrates the construction of YCplac111::ADH1-HAP4, a yeast shuttle vector where HAP4 is expressed from the ADH1 promoter. Plasmids are not drawn to scale.

Figure 8
nucleotide and amino-acid sequence of the ADH1-HAP4 fusion

Figure 9
northern blot analysis of HAP4 overexpression. Dl1 containing YCplac111ADH1 (WT) and DL1 containing YCplac111::ADH1-HAP4 (+HAP4) were grown in medium containing 2% glucose (D) or 2% ethanol/2% glycerol (EG). Total RNA was hybridized with probes specific for HAP4, actin (ACT), QCR8, or SUC2 mRNA.

Figure 10
Biomass yield. Dl1 containing YCplac111::ADH1 (DL1) and DL1 containing YCplac111::ADH1-HAP4 (DL1HAP) were grown in fermentors. Samples for determination of dry weight and glucose concentration were taken with an interval of one hour.

Figure 11
A.Construction of the plasmids p425-GPDHAP4, p425-TEFHAP4,
B.Construction of the plasmids pKSPO2-GPDHAP4 and pKSPO2-TEFHAP4

Figure 12
A. nucleotide and amino acid sequence of the GPD1 promoter fused to the coding region of HAP4.
B. nucleotide and amino acid sequence of the TEF2 promoter fused to the coding region of HAP4.
The fragments shown are the fragments as cloned into pKSPO2 and represent the sequence as integrated in the genome

Figure 13

Scheme of genomic DNA at HAP4 and SIT locus and chromosomal rearrangements after integration of the plasmid pKSPO2-GPD1 or pKSPO2-TEFHAP4 on either the SIT2 locus or the HAP4 locus, and after counterselection on fluoro-acetamide (Fac). Fragments generated after digestion with BamHI or EcoRI which hybridize with the KpnI-XbaI probe (shown as thick black bar) are visualized for comparison with the Southern blots shown in Figure 13 and 15. SITpr: SIT2 promoter, HAP4pr: HAP4 promoter, G/Tpr: GPD or TEF promoter, B: BamHI, E: EcoRI, K: KpnI, X: XbaI, FacR: fluoro-acetamide resistant.

Figure 14

Southern blot of chromosomal DNA digested with BamHI of transformants with pKSPO2-GPDHAP (GH) or pKSPO2-TEFHAP (TH) integrated at the HAP4 locus. The blot was hybridized with the KpnI-XbaI probe shown in Figure 12, visualizing fragments containing SIT2 and/or HAP4 sequences. Radioactivity was visualised and analysed by a Storm 840 Molecular Dynamics Phosphorimager.

Figure 15

Northern blot of total mRNA of transformants with pKSPO2-GPDHAP (GH) or pKSPO2-TEFHAP (TH) integrated at the HAP4 locus as in Figure 14. The blots were hybridized with probes specific for HAP4, QCR8 or PDA1.

Figure 16

Southern blot of chromosomal DNA digested with BamHI or EcoRI of transformants containing pKSPO2-GPDHAP (GH) or pKSPO2-TEFHAP (TH) integrated at the SIT2 locus (A) and three clean transformants containing the GPDHAP4 or TEFHAP4 fusion after counterselection on fluoro-acetamide (B). The blot was hybridized with the KpnI-XbaI probe shown in Figure 12, visualizing fragments containing SIT2 and/or HAP4 sequences.

Figure 17

Northern blot of total mRNA of transformants with pKSPO2-GPDHAP (GH) or pKSPO2-TEFHAP (TH) integrated at the SIT2 locus as in Figure 16. The blots were hybridized with probes specific for HAP4, QCR8 or PDA1 mRNA.

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Table I. Effect of induced expression of HAP4 on transcription

gene	protein	functional HAP 4 binding site	expression in wildtype on glucose	expression in HAP4 - overproducer on glucose
QCR8	11 kD subunit VIII	+	repressed	induced
QCR7	14 kD subunit VII	+	repressed	induced
QCR2	40 kD subunit II	+	repressed	induced
CYC1	iso-1-cytochrome *c*	+	repressed	induced
SUC2	invertase	-	repressed	repressed

Table II. Oxygen consumption capacity of Dl1 and DL1HAP yeast cells

	oxygen consumption nmol/min/mg dry weight	
	Glucose (4%)-grown cells	Lactate-grown cells
DL1	9.4	88.1 (9.4 x wt Glu)
DL1HAP	18.1 (1.9 x wt Glu)	86.7 (9.2 x wt Glu)

Table III. Biomass yield.

strain	Y_{glu} g. dry weight.g^{-1}Glu
Dl1	10.1
DL1HAP	14.8

Table IV. Carbon compounds in culture medium during batch growth in fermentors. Data are mean values of several experiments

strain	ethanol	acetate	glycerol
	$mol.L^{-1}.g^{-1}$ dry weight cells		
Dl1	79.5	1.6	10.2
DL1HAP	49.4	3.6	2.9

Table V. Carbon fluxes indicated as percentage mol C of consumed glucose Carbon balance DL1=103%, DL1HAP=108%

strain	CO_2-TCA	ethanol	acetate	glycerol	biomass
Dl1	7.3	72.5	1.4	9.5	12.2
DL1HAP	15.5	66.0	5.0	4.0	17.8

Table VI. Oxygen consumption capacity of industrial strains

	oxygen consumption nmol/min/mg dry weight	
	Glucose (4%)-grown cells	Lactate-grown cells
DS28911	25.3	ND
DS28911-GH2a	104.0 (4.1x wt glu)	ND
DS28911-TH2a	75.0 (3.0x wt glu)	ND
DS18332	31.5	241 (7.7x wt glu)
DS18332-GH1a	94.5 (3.0x wt glu)	ND
DS18332-TH2a	70.3 (2.2x wt glu)	ND
DS19806	23.8	ND
DS19806-GH1a	87.2 (3.7x wt glu)	ND
DS19806-TH2a	65.6 (2.8x wt glu)	ND

CLAIMS

1. A method for providing a micro-organism which has a preferred metabolic pathway in the presence of a certain carbon source with the capability to inhibit or circumvent said preferred metabolic pathway, comprising providing said micro-organism with the capability to derepress or circumvent the repression of at least one metabolic pathway which is not preferred in the presence of said certain carbon source.

2. A method according to claim 1 whereby the carbon source is glucose.

3. A method according to claim 1 or 2 whereby the micro-organism is a yeast.

4. A method according to claim 3 whereby the yeast is a Saccharomyces.

5. A method according to claim 4 whereby the Saccharomyces is a *Saccharomyces cerevisiae*.

6. A method according to anyone of the aforegoing claims whereby the repressed metabolism of other carbon sources comprises the respiratory pathway or gluconeogenesis.

7. A method according to anyone of the aforegoing claims whereby the repressed metabolism is restored to a significant extent by activation of the pathways for metabolism for the non-preferred carbon sources.

8. A method according to claim 7 whereby said activation is achieved by providing the micro-organism with at least one transcriptional activator for at least one gene encoding an enzyme in said pathways.

9. A method according to claim 8 whereby the transcriptional activator is provided by introduction into the micro-organism of a recombinant nucleic acid encoding said activator.

10. A method according to claim 9 whereby said recombinant nucleic acid is an expression vector.

11. A method according to claim 9 and 10 whereby the recombinant nucleic acid is derived from the same species as the micro-organism.

12. A method according to any one of claims 8-11, whereby the transcriptional activator is constitutively expressed by said micro-organism.

13. A method according to any one of claims 8-11 whereby the transcriptional activator can be expressed by the micro-organism upon induction.

14. A method according to claim 13 whereby expression of said activator is induced by the presence of glucose.

15. A method according to anyone of claims 8-14 whereby the transcriptional activator is a HAP4 protein or a functional equivalent, derivative or fragment thereof.

16. A method according to anyone of the aforegoing claims whereby the micro-organism comprises a recombinant nucleic acid encoding a protein of interest.

17. A method according to claim 16 whereby said recombinant nucleic acid is an expression vector.

18. A method according to claim 16 or 17 whereby said protein of interest is a heterologous protein.

19. A micro-organism obtainable by a method according to anyone of the aforegoing claims.

20. A micro-organism according to claim 19 having improved biomass yield upon culturing.

21. A micro-organism according to claim 19 or 20 showing increased glucose oxidation.

22. A micro-organism according to claim 19, 20 or 21 displaying increased oxidative sugar metabolism.

23. A micro-organism according to any one of claims 19-22 displaying reduced production of ethanol.

24. A micro-organism according to anyone of claims 19-23 which under anaerobic culturing conditions behaves essentially the same as the corresponding micro-organism not provided with the capabilities of anyone of claims 1-18.

GLUCOSE REPRESSED YEAST CELL

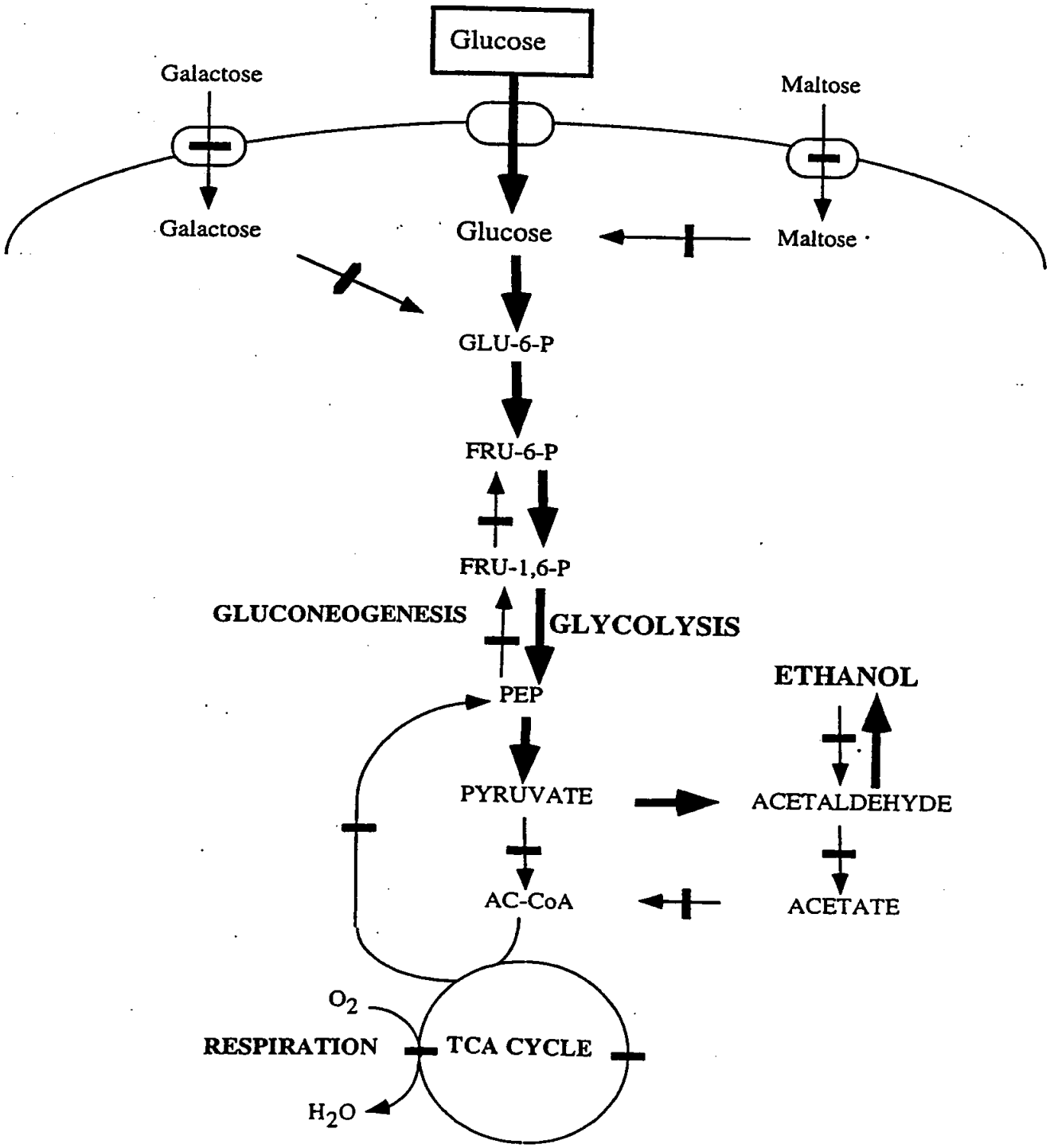


Figure 1 *GLUCOSE REPRESSED/INACTIVATED REACTIONS*

Figure 2

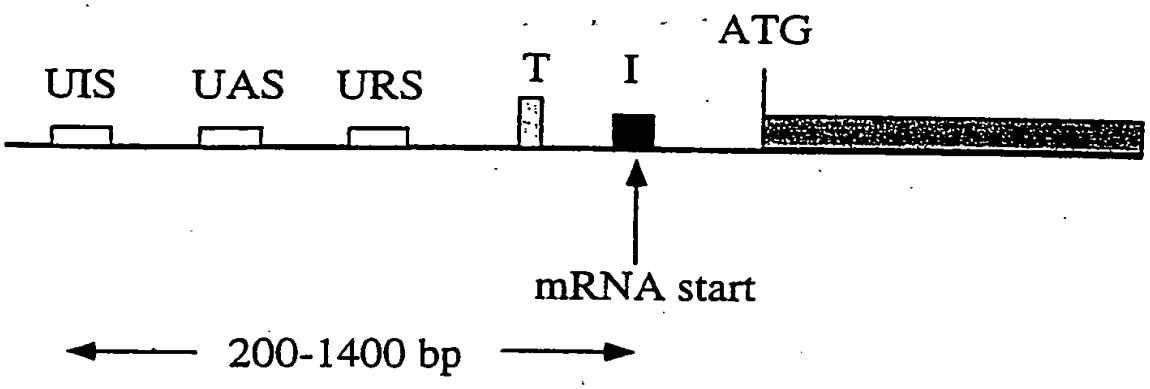


FIG. 2

REGULATORY PATHWAYS INVOLVED IN GLUCOSE REPRESSION

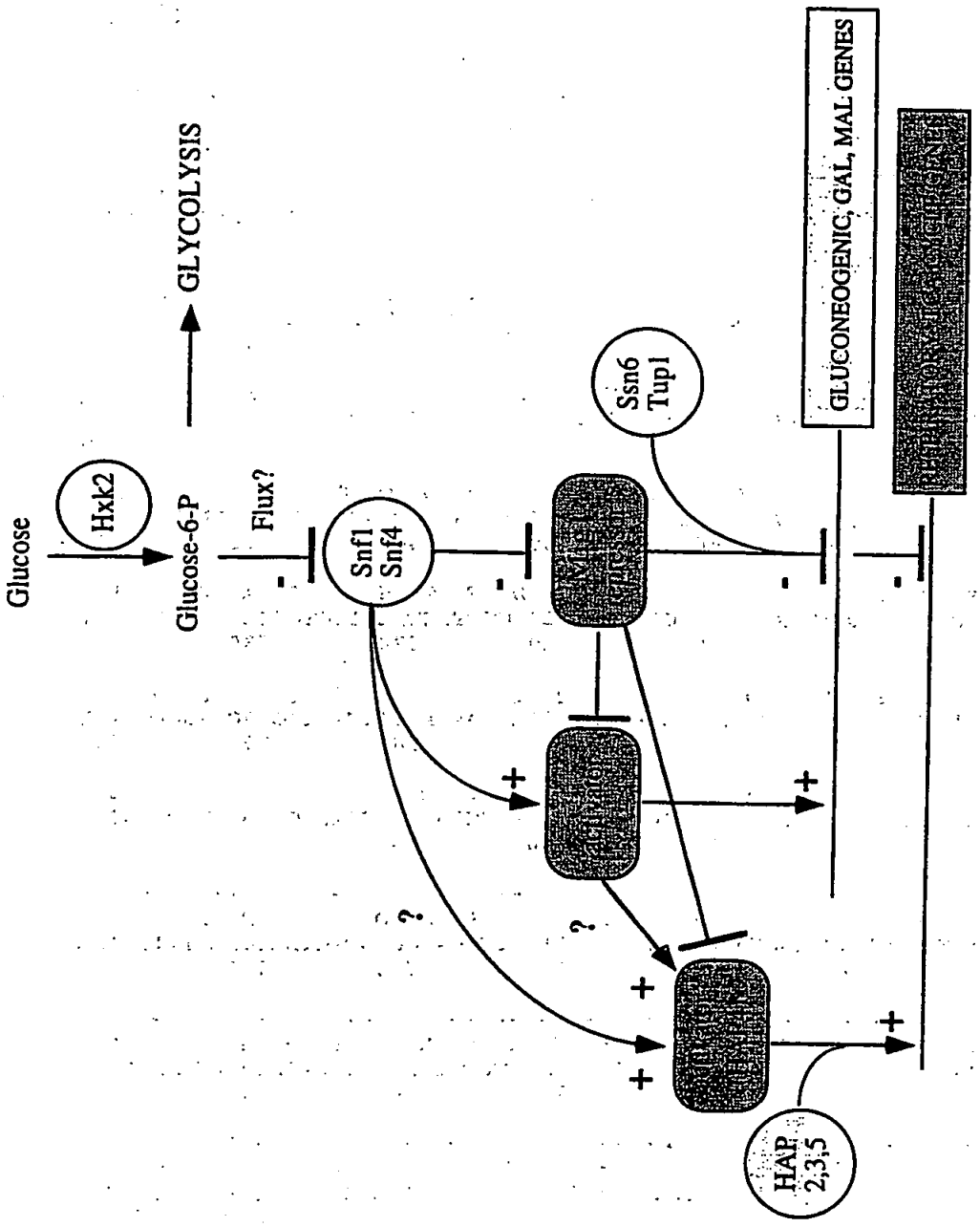


FIG. 3

Figure 4: Nucleotide and amino acid sequence of HAP2

```
TCGTTATTTAGCAATCTTACCCGGAAAACTTCTTGTATCGTAACATTAATTCTCCCTTAC
        10        20        30        40        50        60

AAGGGGACCTCGATGAATAAATAAAGTGTTTGTGTTGAAGGTACTTGCAAAAGGGCAACT
        70        80        90       100       110       120

CACTGTCGTTTATAATTTGATTTACTAATCGCATCTGTATTTGGAAAAGCATTTCTTTTT
       130       140       150       160       170       180

                    M  S  A  D  E  T  D  A  K  F  H  P  L
GGAAGAGGAACAAGAACGCCATGTCAGCAGACGAAACGGATGCGAAATTTCATCCATTAG
       190       200       210       220       230       240

E  T  D  L  Q  S  D  T  A  A  A  T  S  T  A  A  A  S  R  S
AAACAGATCTGCAATCTGATACAGCGGCTGCAACATCAACGGCAGCAGCTTCACGCAGTC
       250       260       270       280       290       300

P  S  L  Q  E  K  P  I  E  M  P  L  D  M  G  K  A  P  S  P
CCTCTCTTCAAGAGAAGCCCATAGAGATGCCCTTGGATATGGGAAAAGCGCCTTCTCCAA
       310       320       330       340       350       360

R  G  E  D  Q  R  V  T  N  E  E  D  L  F  L  F  N  R  L  R
GAGGCGAAGATCAACGGGTTACAAATGAAGAAGATTTGTTTTTGTTTAACAGATTGCGGG
       370       380       390       400       410       420

A  S  Q  N  R  V  M  D  S  L  E  P  Q  Q  Q  S  Q  Y  T  S
CATCACAGAATAGAGTTATGGACTCCTTGGAACCACAACAACAGTCACAGTATACATCTT
       430       440       450       460       470       480

S  S  V  S  T  M  E  P  S  A  D  F  T  S  F  S  A  V  T  T
CCAGTGTCAGTACGATGGAACCATCTGCCGACTTTACTAGTTTCTCTGCAGTGACTACTT
       490       500       510       520       530       540

L  P  P  P  P  H  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q
TACCGCCTCCTCCTCATCAACAACAACAGCAACAACAGCAGCAGCAGCAGCAGCAGCAAT
       550       560       570       580       590       600

L  V  V  Q  A  Q  Y  T  Q  N  Q  P  N  L  Q  S  D  V  L  G
TGGTGGTTCAAGCCCAGTACACCCAAAATCAACCAAACTTGCAAAGCGATGTTTTAGGAA
       610       620       630       640       650       660

                          ----------
T  A  I  A  E  Q  P  F  Y  V  N  A  K  Q  Y  Y  R  I  L  K
CCGCTATAGCAGAGCAACCATTTTATGTTAATGCCAAGCAGTACTACCGAATTTTGAAAA
       670       680       690       700       710       720

------------------------------------------------------------
R  R  Y  A  R  A  K  L  E  E  K  L  R  I  S  R  E  R  K  P
GGCGATATGCAAGAGCTAAACTAGAGGAAAAGCTACGAATATCAAGAGAACGAAAGCCAT
       730       740       750       760       770       780

------------------------------------------------------------
Y  L  H  E  S  R  H  K  H  A  M  R  R  P  R  G  E  G  G  R
ACTTACACGAATCTCGACATAAACATGCGATGCGAAGACCTCGTGGTGAAGGTGGGAGGT
       790       800       810       820       830       840

-------------
F  L  T  A  A  E  I  K  A  M  K  S  K  K  S  G  A  S  D  D
TCTTGACAGCCGCTGAGATCAAAGCCATGAAATCGAAGAAAAGTGGGGCTAGCGATGATC
       850       860       870       880       890       900
```

```
P  D  D  S  H  E  D  K  K  I  T  T  K  I  I  Q  E  Q  P  H
CTGACGATAGTCATGAGGATAAAAAAATCACTACTAAAATAATACAAGAACAGCCGCATG
      910       920       930       940       950       960

A  T  S  T  A  A  A  A  D  K  K  T  *
CTACTTCCACCGCAGCTGCAGCAGACAAAAAAACATAATTTTGTAATATTCCAATGTTAA
      970       980       990      1000      1010      1020

TATCATTCCTAAAAGAACTAAAAGTGCCCTCTTATACCACATGGTATCCATATGGCCTAT
     1030      1040      1050      1060      1070      1080

TTAATCTGAATCAATATGTATATGTACTTTTACCAATCTCGTTTCGTTTCGTTTCGTTTC
     1090      1100      1110      1120      1130      1140

ATTTCTAACAGACCTATGTACTCCGCTGGAAAAGAAACCATATTGCGATCGTATTTAC
     1150      1160      1170      1180      1190
```

Figure 5: Nucleotide and amino acid sequence of HAP3

```
CAAACCTTCTGCCAAAATATAGCACAATAGAAGTACCATATTACGTTCGATGCCACGACA
        10        20        30        40        50        60

ATATCGCGCTACGTGCGTTTTTTGGTCCGCTCTTTCAGACTAAGTAAAAAAAGAGCTGCG
        70        80        90       100       110       120

AATAGTAGCTTTCCGCCAATCAAACTCAAGAGCAGGACTAAGCTAGATAGTAACACAAGT
       130       140       150       160       170       180

                    M  N  T  N  E  S  E  H  V  S  T  S  P
GGCACAAACCTCTCGAGAATATGAATACCAACGAGTCCGAACATGTTAGCACAAGCCCAG
       190       200       210       220       230       240

E  D  T  Q  E  N  G  G  N  A  S  S  S  G  S  L  Q  Q  I  S
AGGATACTCAGGAGAACGGTGGAAACGCTAGCTCCAGCGGCAGTTTGCAGCAAATTTCCA
       250       260       270       280       290       300

      ------------------------------------------------
T  L  R  E  Q  D  R  W  L  P  I  N  N  V  A  R  L  M  K  N
CGCTAAGAGAGCAGGACAGATGGCTACCCATCAACAATGTAGCGCGACTCATGAAGAATA
       310       320       330       340       350       360

------------------------------------------------------------
T  L  P  P  S  A  K  V  S  K  D  A  K  E  C  M  Q  E  C  V
CTCTCCCACCGAGTGCTAAGGTATCGAAAGATGCGAAAGAGTGCATGCAGGAGTGTGTCA
       370       380       390       400       410       420

------------------------------------------------------------
S  E  L  I  S  F  V  T  S  E  A  S  D  R  C  A  A  D  K  R
GTGAGCTCATTTCTTTTGTGACTAGCGAGGCCAGCGATCGATGCGCTGCTGACAAAAGAA
       430       440       450       460       470       480

------------------------------------------------------------
K  T  I  N  G  E  D  I  L  I  S  L  H  A  L  G  F  E  N  Y
AGACGATAAACGGGGAAGACATTCTCATATCATTGCACGCCTTAGGATTCGAGAACTATG
       490       500       510       520       530       540

-----------------------------------
A  E  V  L  K  I  Y  L  A  K  Y  R  Q  Q  Q  A  L  K  N  Q
CAGAGGTGTTGAAAATCTACTTGGCTAAATACAGGCAACAACAGGCGCTGAAGAATCAAC
       550       560       570       580       590       600

L  M  Y  E  Q  D  D  E  E  V  P  *
TAATGTATGAGCAGGACGACGAAGAGGTGCCTTGAGAAGACAAAACCAGGTGGTAGATCG
       610       620       630       640       650       660

CAAAAGTTGCTAGCTGTCAGGATGGAATAGCACGGGGCTATTTCCTGCTGGTCGTTGGTT
       670       680       690       700       710       720

CTCGTGTAATTAATGAATGTAACGATATAGATAATATTTTATTGTTAGTGTGTAATGTAT
       730       740       750       760       770       780

TCAATGTAATGTATGGGTGCTTTGTAAAGGGTGTATGATGTTTGCCACCGGAAGG
       790       800       810       820       830
```

Figure 6: Nucleotide and amino acid sequence of HAP4

```
                                                                      TAAAGGAACCAGAAAAATAAAAAAGGGTCATTATTTATTTGAGCAGATCATTATCAAACG
                                                                      10        20        30        40        50        60

CATAGGAAGAGAAAAAACACAGTTTTATTTTTTTTCCACACATATTTATTGGTCTCCTAG
70        80        90        100       110       120

TACATCAAAGAGCATTTTAATGGGTTGCTGATTTGTTTTACCTACATTTTCTAGTACAAA
130       140       150       160       170       180

                                  M  T  A  K  T  F  L  L  Q  A  S  A  S
AAAAAAACAAAAAAAGAATCATGACCGCAAAGACTTTTCTACTACAGGCCTCCGCTAGTC
190       200       210       220       230       240

R  P  R  S  N  H  F  K  N  E  H  N  N  I  P  L  A  P  V  P
GCCCTCGTAGTAACCATTTTAAAAATGAGCATAATAATATTCCATTGGCGCCTGTACCGA
250       260       270       280       290       300

I  A  P  N  T  N  H  H  N  N  S  S  L  E  F  E  N  D  G  S
TCGCCCCAAATACCAACCATCATAACAATAGTTCGCTGGAATTCGAAAACGATGGCAGTA
310       320       330       340       350       360

K  K  K  K  K  S  S  L  V  V  R  T  S  K  H  W  V  L  P  P
AAAAGAAGAAGAAGTCTAGCTTGGTGGTTAGAACTTCAAAACATTGGGTTTTGCCCCCAA
370       380       390       400       410       420

R  P  R  P  G  R  R  S  S  S  H  N  T  L  P  A  N  N  T  N
GACCAAGACCTGGTAGAAGATCATCTTCTCACAACACTCTACCTGCCAACAACACCAATA
430       440       450       460       470       480

N  I  L  N  V  G  P  N  S  R  N  S  S  N  N  N  N  N  N  N
ATATTTTAAATGTTGGCCCTAACAGCAGGAACAGTAGTAATAATAATAATAATAATAACA
490       500       510       520       530       540

I  I  S  N  R  K  Q  A  S  K  E  K  R  K  I  P  R  H  I  Q
TCATTTCGAATAGGAAACAAGCTTCCAAAGAAAAGAGGAAAATACCAAGACATATCCAGA
550       560       570       580       590       600

T  I  D  E  K  L  I  N  D  S  N  Y  L  A  F  L  K  F  D  D
CAATCGATGAAAAGCTAATAAACGACTCGAATTACCTCGCATTTTTGAAGTTCGATGACT
610       620       630       640       650       660

L  E  N  E  K  F  H  S  S  A  S  S  I  S  S  P  S  Y  S  S
TGGAAAATGAAAAGTTTCATTCTTCTGCCTCCTCCATTTCATCTCCATCTTATTCATCTC
670       680       690       700       710       720

P  S  F  S  S  Y  R  N  R  K  K  S  E  F  M  D  D  E  S  C
CATCTTTTTCAAGTTATAGAAATAGAAAAAAATCAGAATTCATGGACGATGAAAGCTGCA
730       740       750       760       770       780

T  D  V  E  T  I  A  A  H  N  S  L  L  T  K  N  H  H  I  D
CCGATGTGGAAACCATTGCTGCTCACAACAGTCTGCTAACAAAAAACCATCATATAGATT
790       800       810       820       830       840

S  S  S  N  V  H  A  P  P  T  K  K  S  K  L  N  D  F  D  L
CTTCTTCAAATGTTCACGCACCACCCACGAAAAAATCAAAGTTGAACGACTTTGATTTAT
850       860       870       880       890       900

L  S  L  S  S  T  S  S  S  A  T  P  V  P  Q  L  T  K  D  L
TGTCCTTATCTTCCACATCTTCATCGGCCACTCCGGTCCCACAGTTGACAAAAGATTTGA
910       920       930       940       950       960
```

```
N  M  N  L  N  F  H  K  I  P  H  K  A  S  F  P  D  S  P  A
ACATGAACCTAAATTTTCATAAGATCCCTCATAAGGCTTCATTCCCTGATTCTCCAGCAG
      970        980        990       1000       1010       1020

D  F  S  P  A  D  S  V  S  L  I  R  N  H  S  L  P  T  N  L
ATTTCTCTCCAGCAGATTCAGTCTCGTTGATTAGAAACCACTCCTTGCCTACTAATTTGC
      1030       1040       1050       1060       1070       1080

Q  V  K  D  K  I  E  D  L  N  E  I  K  F  F  N  D  F  E  K
AAGTTAAGGACAAAATTGAGGATTTGAACGAGATTAAATTCTTTAACGATTTCGAGAAAC
      1090       1100       1110       1120       1130       1140

L  E  F  F  N  K  Y  A  K  V  N  T  N  N  D  V  N  E  N  N
TTGAGTTTTTCAATAAGTATGCCAAAGTCAACACGAATAACGACGTTAACGAAAATAATG
      1150       1160       1170       1180       1190       1200

D  L  W  N  S  Y  L  Q  S  M  D  D  T  T  G  K  N  S  G  N
ATCTCTGGAATTCTTACTTACAGTCTATGGACGATACAACAGGTAAGAACAGTGGCAATT
      1210       1220       1230       1240       1250       1260

Y  Q  Q  V  D  N  D  D  N  M  S  L  L  N  L  P  I  L  E  E
ACCAACAAGTGGACAATGACGATAATATGTCTTTATTGAATCTGCCAATTTTGGAGGAAA
      1270       1280       1290       1300       1310       1320

T  V  S  S  G  Q  D  D  K  V  E  P  D  E  E  D  I  W  N  Y
CCGTATCTTCAGGGCAAGATGATAAGGTTGAGCCAGATGAAGAAGACATTTGGAATTATT
      1330       1340       1350       1360       1370       1380

L  P  S  S  S  S  Q  Q  E  D  S  S  R  A  L  K  K  N  T  N
TACCAAGTTCAAGTTCACAACAAGAAGATTCATCACGTGCTTTGAAAAAAAATACTAATT
      1390       1400       1410       1420       1430       1440

                                                  ----------
S  E  K  A  N  I  Q  A  K  N  D  E  T  Y  L  F  L  Q  D  Q
CTGAGAAGGCGAACATCCAAGCAAAGAACGATGAAACCTATCTGTTTCTTCAGGATCAGG
      1450       1460       1470       1480       1490       1500

------------------------------------------------------------
D  E  S  A  D  S  H  H  H  D  E  L  G  S  E  I  T  L  A  D
ATGAAAGCGCTGATTCGCATCACCATGACGAGTTAGGTTCAGAAATCACTTTGGCTGACA
      1510       1520       1530       1540       1550       1560

------------------------------------------------------------
N  K  F  S  Y  L  P  P  T  L  E  E  L  M  E  E  Q  D  C  N
ATAAGTTTTCTTATTTGCCCCCAACTCTAGAAGAGTTGATGGAAGAGCAGGACTGTAACA
      1570       1580       1590       1600       1610       1620

N  G  R  S  F  K  N  F  M  F  S  N  D  T  G  I  D  G  S  A
ATGGCAGATCTTTTAAAAATTTCATGTTTTCCAACGATACCGGTATTGACGGTAGTGCCG
      1630       1640       1650       1660       1670       1680

G  T  D  D  D  Y  T  K  V  L  K  S  K  K  I  S  T  S  K  S
GTACTGATGACGACTACACCAAAGTTCTGAAATCCAAAAAAATTTCTACGTCGAAGTCGA
      1690       1700       1710       1720       1730       1740

                        ----------------------------------------
N  A  N  L  Y  D  L  N  D  N  N  N  D  A  T  A  T  N  E  L
ACGCTAACCTTTATGACTTAAACGATAACAACAATGATGCAACTGCCACCAATGAACTTG
      1750       1760       1770       1780       1790       1800

------------------------------------------------
D  Q  S  S  F  I  D  D  L  D  E  D  V  D  F  L  K  V  Q  V
ATCAAAGCAGTTTCATCGACGACCTTGACGAAGATGTCGATTTTTTAAAGGTACAAGTAT
```

```
      1810        1820        1830        1840        1850        1860
F  *
TTTGAAATAGGCATGTTGCAATAAAACGAAAACAACTAAAAATCACGAAAACAAAATGAT
      1870        1880        1890        1900        1910        1920
ATTATACAATAAAAAATTCTTATTATGGGTAATGATAGTATTCTTCGCCTGCTTAGGCGT
      1930        1940        1950        1960        1970        1980
CCTTTTCCTTCAACAACAAAAATTCCAAAAAAAAAAAAGTAAAAAAACAAAACTTTGATTG
      1990        2000        2010        2020        2030        2040
TTTTTTAATGATGTTAATGATTTTT
      2050        2060
```

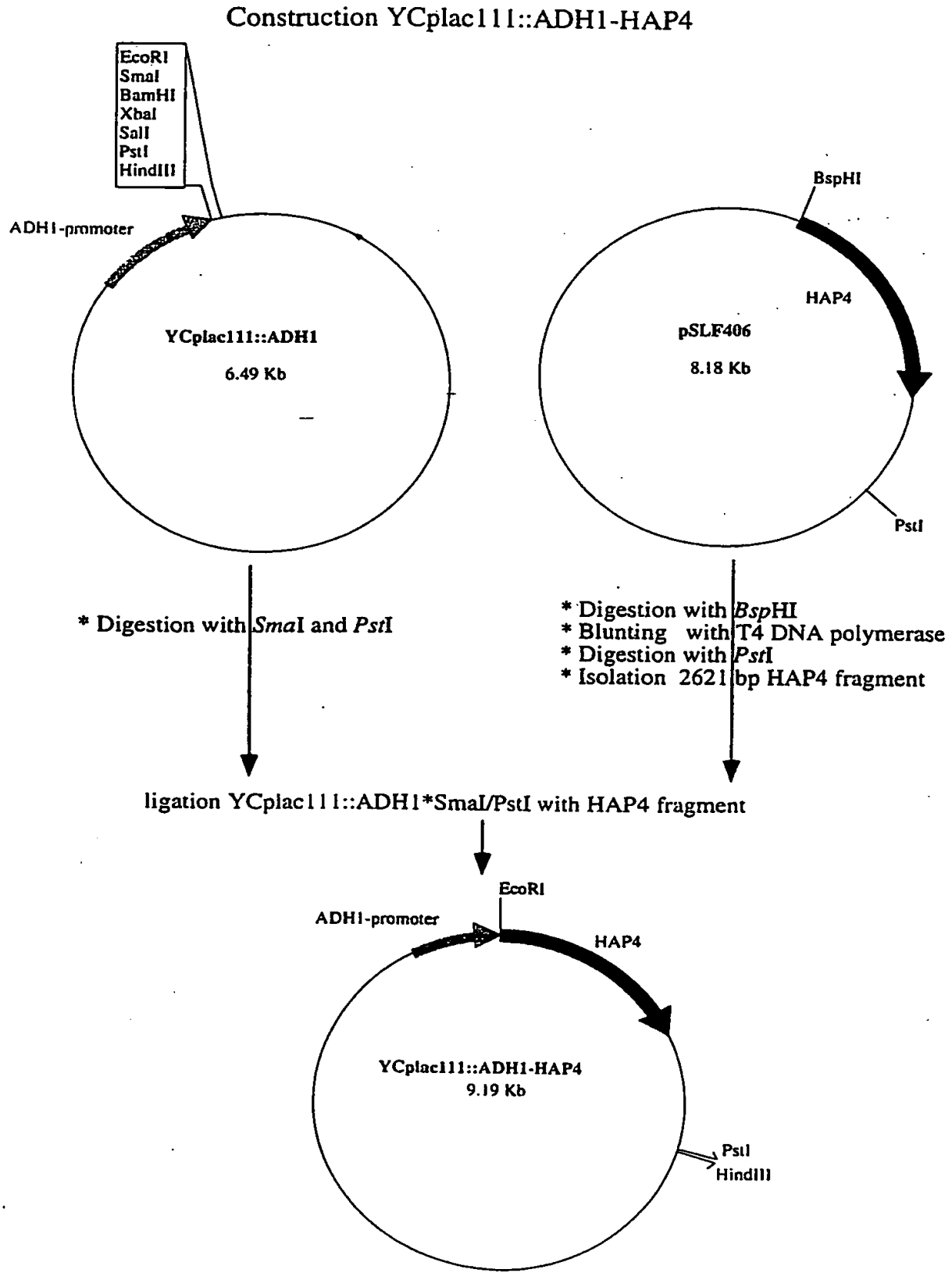

Construction YCplac111::ADH1-HAP4
EcoRI
SmaI
BamHI
XbaI
SalI
PstI
HindIII
ADH1-promoter
YCplac111::ADH1
6.49 Kb
BspHI
HAP4
pSLF406
8.18 Kb
PstI
* Digestion with SmaI and PstI
* Digestion with BspHI
* Blunting with T4 DNA polymerase
* Digestion with PstI
* Isolation 2621 bp HAP4 fragment
ligation YCplac111::ADH1*SmaI/PstI with HAP4 fragment
EcoRI
ADH1-promoter
HAP4
YCplac111::ADH1-HAP4
9.19 Kb
PstI
HindIII

Figure 7

Figure 8: Nucleotide and amino acid sequence of the ADH1 promoter fused to the coding region of HAP4

```
atccttttgttgtttccgggtgtacaatatggacttcctcttttctggcaaccaaaccca
          10        20        30        40        50
60

tacatcgggattcctataataccttcgttggtctccctaacatgtaggtggcggagggga
          70        80        90       100       110
120

gatatacaatagaacagataccagacaagacataatgggctaaacaagactacaccaatt
         130       140       150       160       170
180

acactgcctcattgatggtggtacataacgaactaatactgtagccctagacttgatagc
         190       200       210       220       230
240

catcatcatatcgaagtttcactacccttttccatttgccatctattgaagtaataata
         250       260       270       280       290
300

ggcgcatgcaacttcttttcttttttttttcttttctctctcccccgttgttgtctcacca
         310       320       330       340       350
360

tatccgcaatgacaaaaaaaatgatggaagacactaaaggaaaaaattaacgacaaagac
         370       380       390       400       410
420

agcaccaacagatgtcgttgttccagagctgatgaggggtatcttcgaacacacgaaact
         430       440       450       460       470
480

ttttccttccttcattcacgcacactactctctaatgagcaacggtatacggccttcctt
         490       500       510       520       530
540

ccagttacttgaatttgaaataaaaaaagtttgccgctttgctatcaagtataaatagac
         550       560       570       580       590
600

ctgcaattattaatcttttgtttcctcgtcattgttctcgttccctttcttccttgtttc
```

610 620 630 640 650
660

ttttctgcacaatatttcaagctataccaagcatacaatcaaggaattcgagctcgccc
670 680 690 700 710
720

M T A K T F L L Q A S A S R P R S N H
F

cATGACCGCAAAGACTTTTCTACTACAGGCCTCCGCTAGTCGCCCTCGTAGTAACCATTT
730 740 750 760 770
780

K N E H N N I P L A P V P I A P N T N
H

TAAAAATGAGCATAATAATATTCCATTGGCGCCTGTACCGATCGCCCCAAATACCAACCA
790 800 810 820 830
840

H N N S S L E F E N D G S K K K K K S
S

TCATAACAATAGTTCGCTGGAATTCGAAAACGATGGCAGTAAAAAGAAGAAGAAGTCTAG
850 860 870 880 890
900

L V V R T S K H W V L P P R P R P G R
R

CTTGGTGGTTAGAACTTCAAAACATTGGGTTTTGCCCCCAAGACCAAGACCTGGTAGAAG
910 920 930 940 950
960

S S S H N T L P A N N T N N I L N V G
P

ATCATCTTCTCACAACACTCTACCTGCCAACAACACCAATAATATTTTAAATGTTGGCCC
970 980 990 1000 1010
1020

N S R N S S N N N N N N N I I S N R K
Q

TAACAGCAGGAACAGTAGTAATAATAATAATAATAACATCATTTCGAATAGGAAACA
1030 1040 1050 1060 1070
1080

A S K E K R K I P R H I Q T I D E K L
I

AGCTTCCAAAGAAAAGAGGAAAATACCAAGACATATCCAGACAATCGATGAAAAGCTAAT
1090 1100 1110 1120 1130
1140

```
        N  D  S  N  Y  L  A  F  L  K  F  D  D  L  E  N  E  K  F
R

AAACGACTCGAATTACCTCGCATTTTTGAAGTTCGATGACTTGGAAAATGAAAAGTTTCG
          1150       1160       1170       1180       1190
1200

        S  S  A  S  S  I  S  S  P  S  Y  S  S  P  S  F  S  S  Y
R

TTCTTCTGCCTCCTCCATTTCATCTCCATCTTATTCATCTCCATCTTTTTCAAGTTATAG
          1210       1220       1230       1240       1250
1260

        N  R  K  K  S  E  F  M  D  D  E  S  C  T  D  V  E  T  I
A

AAATAGAAAAAAATCAGAATTCATGGACGATGAAAGCTGCACCGATGTGGAAACCATTGC
          1270       1280       1290       1300       1310
1320

        A  H  N  S  L  L  T  K  N  H  H  I  D  S  S  S  N  V  H
A

TGCTCACAACAGTCTGCTAACAAAAAACCATCATATAGATTCTTCTTCAAATGTTCACGC
          1330       1340       1350       1360       1370
1380

        P  P  T  K  K  S  K  L  N  D  F  D  L  L  S  L  S  S  T
S

ACCACCCACGAAAAAATCAAAGTTGAACGACTTTGATTTATTGTCCTTATCTTCCACATC
          1390       1400       1410       1420       1430
1440

        S  S  A  T  P  V  P  Q  L  T  K  D  L  N  M  N  L  N  F
H

TTCATCGGCCACTCCGGTCCCACAGTTGACAAAAGATTTGAACATGAACCTAAATTTTCA
          1450       1460       1470       1480       1490
1500

        K  I  P  H  K  A  S  F  P  D  S  P  A  D  F  S  P  A  D
S

TAAGATCCCTCATAAGGCTTCATTCCCTGATTCTCCAGCAGATTTCTCTCCAGCAGATTC
          1510       1520       1530       1540       1550
1560

        V  S  L  I  R  N  H  S  L  P  T  N  L  Q  V  K  D  K  I
E

AGTCTCGTTGATTAGAAACCACTCCTTGCCTACTAATTTGCAAGTTAAGGACAAAATTGA
          1570       1580       1590       1600       1610
1620

        D  L  N  E  I  K  F  F  N  D  F  E  K  L  E  F  F  N  K
Y
```

```
GGATTTGAACGAGATTAAATTCTTTAACGATTTCGAGAAACTTGAGTTTTTCAATAAGTA
          1630       1640       1650       1660       1670
1680

      A  K  V  N  T  N  N  D  V  N  E  N  N  D  L  W  N  S  Y
L

TGCCAAAGTCAACACGAATAACGACGTTAACGAAAATAATGATCTCTGGAATTCTTACTT
          1690       1700       1710       1720       1730
1740

      Q  S  M  D  D  T  T  G  K  N  S  G  N  Y  Q  Q  V  D  N
D

ACAGTCTATGGACGATACAACAGGTAAGAACAGTGGCAATTACCAACAAGTGGACAATGA
          1750       1760       1770       1780       1790
1800

      D  N  M  S  L  L  N  L  P  I  L  E  E  T  V  S  S  G  Q
D

CGATAATATGTCTTTATTGAATCTGCCAATTTTGGAGGAAACCGTATCTTCAGGGCAAGA
          1810       1820       1830       1840       1850
1860

      D  K  V  E  P  D  E  E  D  I  W  N  Y  L  P  S  S  S  S
Q

TGATAAGGTTGAGCCAGATGAAGAAGACATTTGGAATTATTTACCAAGTTCAAGTTCACA
          1870       1880       1890       1900       1910
1920

      Q  E  D  S  S  R  A  L  K  K  N  T  N  S  E  K  A  N  I
Q

ACAAGAAGATTCATCACGTGCTTTGAAAAAAAATACTAATTCTGAGAAGGCGAACATCCA
          1930       1940       1950       1960       1970
1980

      A  K  N  D  E  T  Y  L  F  L  Q  D  Q  D  E  S  A  D  S
H

AGCAAAGAACGATGAAACCTATCTGTTTCTTCAGGATCAGGATGAAAGCGCTGATTCGCA
          1990       2000       2010       2020       2030
2040

      H  H  D  E  L  G  S  E  I  T  L  A  D  N  K  F  S  Y  L
P

TCACCATGACGAGTTAGGTTCAGAAATCACTTTGGCTGACAATAAGTTTTCTTATTTGCC
          2050       2060       2070       2080       2090
2100

      P  T  L  E  E  L  M  E  E  Q  D  C  N  N  G  R  S  F  K
N

CCCAACTCTAGAAGAGTTGATGGAAGAGCAGGACTGTAACAATGGCAGATCTTTTAAAAA
```

```
                2110        2120        2130        2140        2150
2160
          F   M   F   S   N   D   T   G   I   D   G   S   A   G   T   D   D   D   Y
T
TTTCATGTTTTCCAACGATACCGGTATTGACGGTAGTGCCGGTACTGATGACGACTACAC
                2170        2180        2190        2200        2210
2220
          K   V   L   K   S   K   K   I   S   T   S   K   S   N   A   N   L   Y   D
L
CAAAGTTCTGAAATCCAAAAAAATTTCTACGTCGAAGTCGAACGCTAACCTTTATGACTT
                2230        2240        2250        2260        2270
2280
          N   D   N   N   N   D   A   T   A   T   N   E   L   D   Q   S   S   F   I
D
AAACGATAACAACAATGATGCAACTGCCACCAATGAACTTGATCAAAGCAGTTTCATCGA
                2290        2300        2310        2320        2330
2340
          D   L   D   E   D   V   D   F   L   K   V   Q   V   F   *
CGACCTTGACGAAGATGTCGATTTTTTAAAGGTACAAGTATTTTAAggggatcc
                2350        2360        2370        2380        2390
```

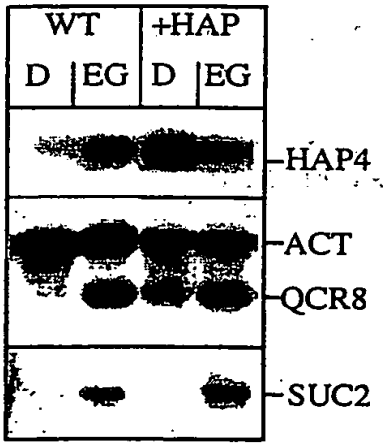


Figure 9

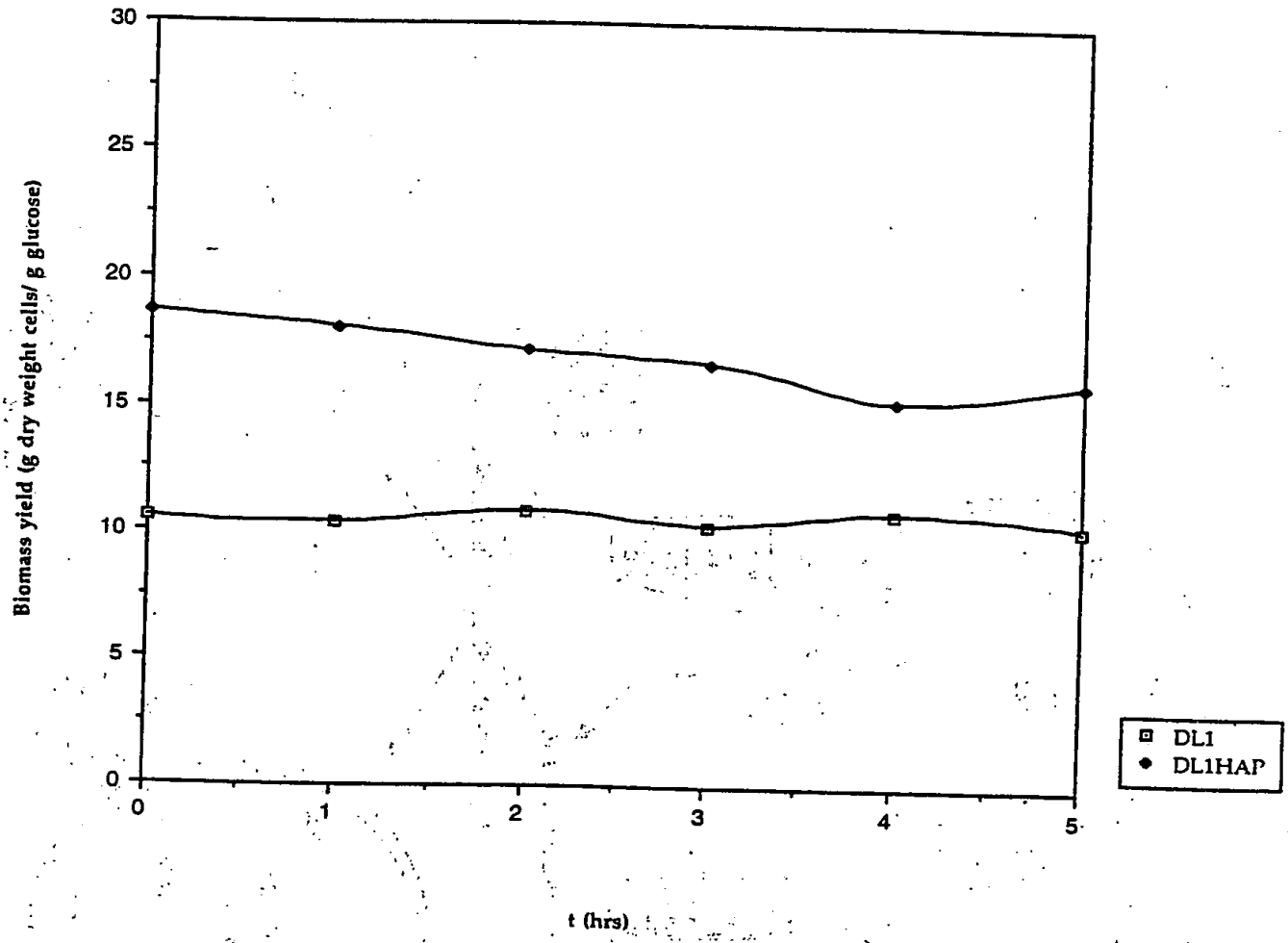

30
25
20
15
10
5
0
0
1
2
3
4
5
Biomass yield (g dry weight cells/ g glucose)
t (hrs)
DL1
DL1HAP

Figure 10

Figure 11A

Construction p425-GPDHAP4 and p425-TEFHAP4

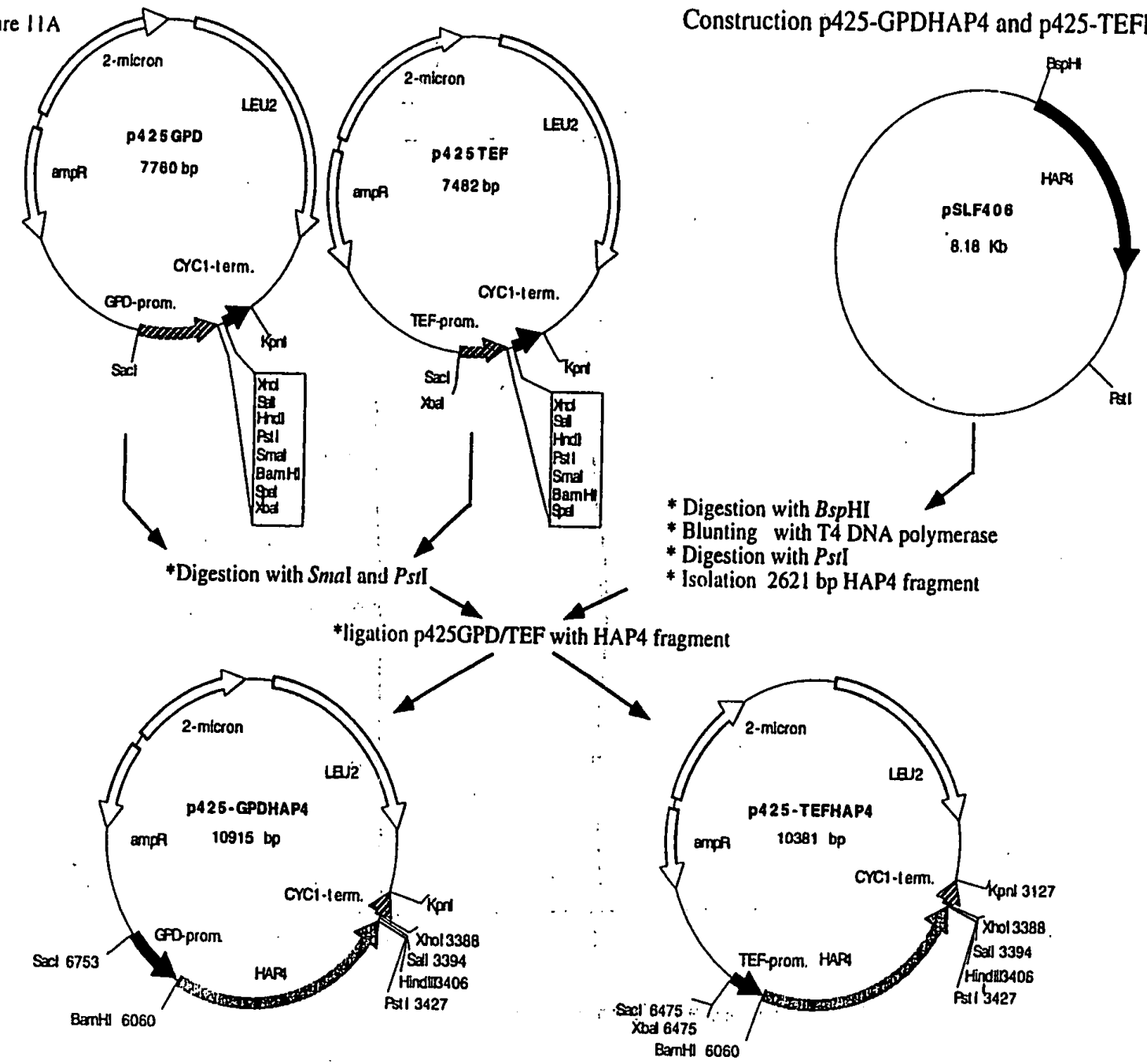


Figure 11B Construction pKSPO2-GPDHAP4 and pKSPO2-TEFHAP4

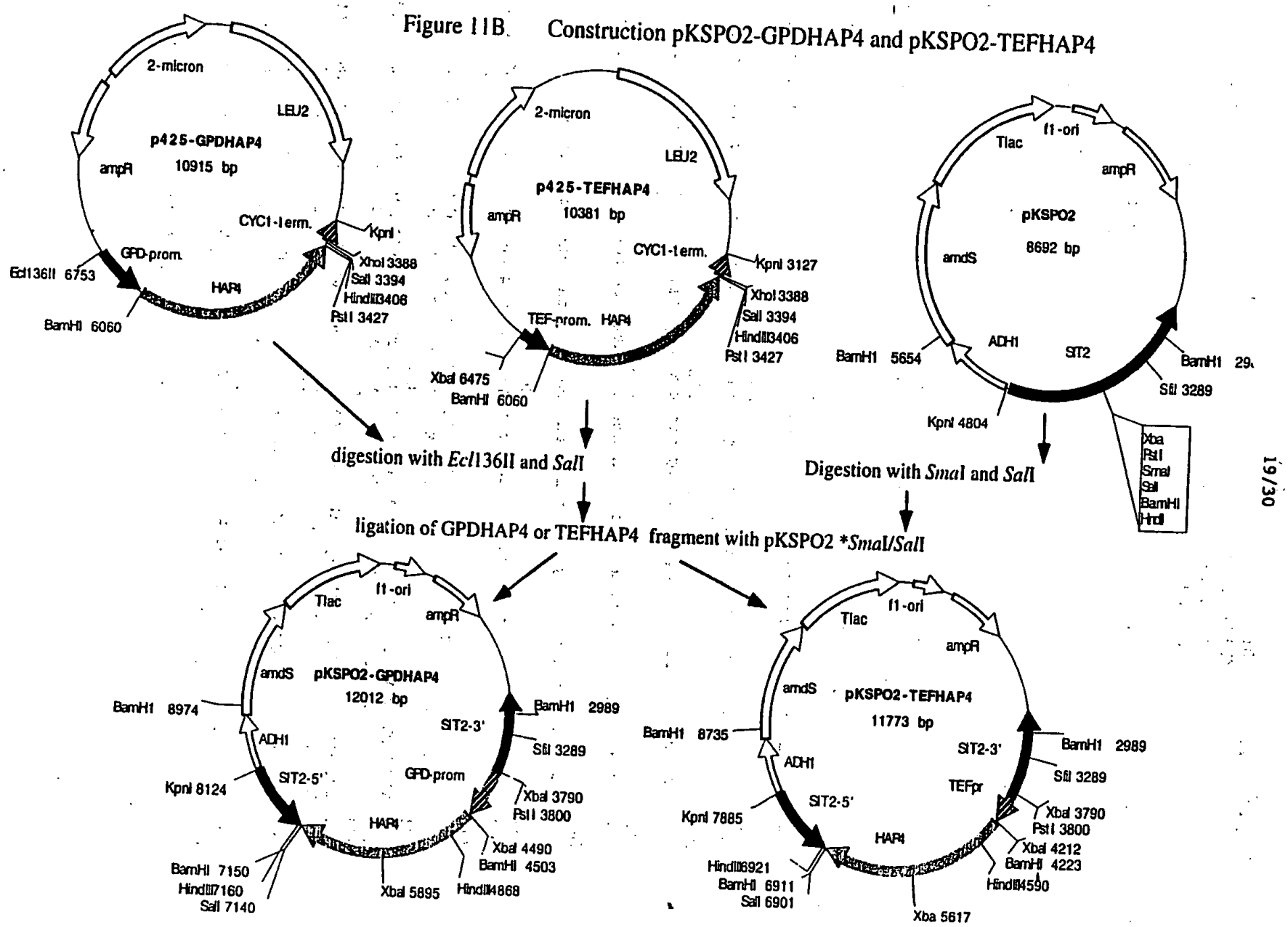


Figure 12A. Nucleotide and amino acid sequence of the 680 bp GPD1 promoter fused to the HAP4 coding region, as cloned in pKSPO2 and integrated in the yeast genome

```
1                                                   31
gag ctc tgg ggt ttg agc aag tct aag ttt acg tag cat aaa aat tct cgg att gcg tca

61                                                  91
aat aat aaa aaa agt aac ccc act tct act tct aca tcg gaa aaa cat tcc att cac ata

121                                                 151
tcg tct ttg gcc tat ctt gtt ttg tcc tcg gta gat cag gtc agt aca aac gca aca cga

181                                                 211
aag aac aaa aaa aga aga aaa cag aag gcc aag aca ggg tca atg aga ctg ttg tcc tcc

241                                                 271
tac tgt ccc tat gtc tct ggc cga tca cgc gcc att gtc cct cag aaa caa atc aaa cac

301                                                 331
cca cac ccc ggg cac cca aag tcc cca ccc aca cca cca ata cgt aaa cgg ggc gcc ccc

361                                                 391
tgc agg ccc tcc tgc gcg cgg cct ccc gcc ttg ctt ctc tcc cct tcc ttt tct ttt tcc

421                                                 451
agt ttt ccc tat ttt gtc cct ttt tcc gca caa caa gta tca gaa tgg gtt cat caa atc

481                                                 511
tat cca acc taa ttc gca cgt aga ctg gct tgg tat tgg cag ttt cgt agt tat ata tat

541                                                 571
act acc atg agt gaa act gtt acg tta cct taa att ctt tct ccc ttt aat ttt ctt tta

601                                                 631
tct tac tct cct aca taa gac atc aag aaa caa ttg tat att gta cac ccc ccc cct cca

661                                                 691
caa aca caa ata ttg ata ata taa agt cta gaa cta gtg gat ccc ccc ATG ACC GCA AAG
                                                                M   T   A   K
721                                                 751
ACT TTT CTA CTA CAG GCC TCC GCT AGT CGC CCT CGT AGT AAC CAT TTT AAA AAT GAG CAT
T   F   L   L   Q   A   S   A   S   R   P   R   S   N   H   F   K   N   E   H
781                                                 811
AAT AAT ATT CCA TTG GCG CCT GTA CCG ATC GCC CCA AAT ACC AAC CAT CAT AAC AAT AGT
N   N   I   P   L   A   P   V   P   I   A   P   N   T   N   H   H   N   N   S
841                                                 871
TCG CTG GAA TTC GAA AAC GAT GGC AGT AAA AAG AAG AAG AAG TCT AGC TTG GTG GTT AGA
S   L   E   F   E   N   D   G   S   K   K   K   K   S   S   L   V   V   R
901                                                 931
ACT TCA AAA CAT TGG GTT TTG CCC CCA AGA CCA AGA CCT GGT AGA AGA TCA TCT TCT CAC
T   S   K   H   W   V   L   P   P   R   P   R   P   G   R   R   S   S   S   H
961                                                 991
AAC ACT CTA CCT GCC AAC AAC ACC AAT AAT ATT TTA AAT GTT GGC CCT AAC AGC AGG AAC
N   T   L   P   A   N   N   T   N   N   I   L   N   V   G   P   N   S   R   N
1021                                                1051
AGT AGT AAT AAT AAT AAT AAT AAT AAC ATC ATT TCG AAT AGG AAA CAA GCT TCC AAA GAA
```

```
S   S   N   N   N   N   N   N   N   I   I   S   N   R   K   Q   A   S   K   E
1081                                                1111
AAG AGG AAA ATA CCA AGA CAT ATC CAG ACA ATC GAT GAA AAG CTA ATA AAC GAC TCG AAT
K   R   K   I   P   R   H   I   Q   T   I   D   E   K   L   I   N   D   S   N
1141                                                1171
TAC CTC GCA TTT TTG AAG TTC GAT GAC TTG GAA AAT GAA AAG TTT CAT TCT TCT GCC TCC
Y   L   A   F   L   K   F   D   D   L   E   N   E   K   F   H   S   S   A   S
1201                                                1231
TCC ATT TCA TCT CCA TCT TAT TCA TCT CCA TCT TTT TCA AGT TAT AGA AAT AGA AAA AAA
S   I   S   S   P   S   Y   S   S   P   S   F   S   S   Y   R   N   R   K   K
1261                                                1291
TCA GAA TTC ATG GAC GAT GAA AGC TGC ACC GAT GTG GAA ACC ATT GCT GCT CAC AAC AGT
S   E   F   M   D   D   E   S   C   T   D   V   E   T   I   A   A   H   N   S
1321                                                1351
CTG CTA ACA AAA AAC CAT CAT ATA GAT TCT TCT TCA AAT GTT CAC GCA CCA CCC ACG AAA
L   L   T   K   N   H   H   I   D   S   S   S   N   V   H   A   P   P   T   K
1381                                                1411
AAA TCA AAG TTG AAC GAC TTT GAT TTA TTG TCC TTA TCT TCC ACA TCT TCA TCG GCC ACT
K   S   K   L   N   D   F   D   L   L   S   L   S   S   T   S   S   S   A   T
1441                                                1471
CCG GTC CCA CAG TTG ACA AAA GAT TTG AAC ATG AAC CTA AAT TTT CAT AAG ATC CCT CAT
P   V   P   Q   L   T   K   D   L   N   M   N   L   N   F   H   K   I   P   H
1501                                                1531
AAG GCT TCA TTC CCT GAT TCT CCA GCA GAT TTC TCT CCA GCA GAT TCA GTC TCG TTG ATT
K   A   S   F   P   D   S   P   A   D   F   S   P   A   D   S   V   S   L   I
1561                                                1591
AGA AAC CAC TCC TTG CCT ACT AAT TTG CAA GTT AAG GAC AAA ATT GAG GAT TTG AAC GAG
R   N   H   S   L   P   T   N   L   Q   V   K   D   K   I   E   D   L   N   E
1621                                                1651
ATT AAA TTC TTT AAC GAT TTC GAG AAA CTT GAG TTT TTC AAT AAG TAT GCC AAA GTC AAC
I   K   F   F   N   D   F   E   K   L   E   F   F   N   K   Y   A   K   V   N
1681                                                1711
ACG AAT AAC GAC GTT AAC GAA AAT AAT GAT CTC TGG AAT TCT TAC TTA CAG TCT ATG GAC
T   N   N   D   V   N   E   N   N   D   L   W   N   S   Y   L   Q   S   M   D
1741                                                1771
GAT ACA ACA GGT AAG AAC AGT GGC AAT TAC CAA CAA GTG GAC AAT GAC GAT AAT ATG TCT
D   T   T   G   K   N   S   G   N   Y   Q   Q   V   D   N   D   D   N   M   S
1801                                                1831
TTA TTG AAT CTG CCA ATT TTG GAG GAA ACC GTA TCT TCA GGG CAA GAT GAT AAG GTT GAG
L   L   N   L   P   I   L   E   E   T   V   S   S   G   Q   D   D   K   V   E
1861                                                1891
CCA GAT GAA GAA GAC ATT TGG AAT TAT TTA CCA AGT TCA AGT TCA CAA CAA GAA GAT TCA
P   D   E   E   D   I   W   N   Y   L   P   S   S   S   S   Q   Q   E   D   S
1921                                                1951
TCA CGT GCT TTG AAA AAA AAT ACT AAT TCT GAG AAG GCG AAC ATC CAA GCA AAG AAC GAT
S   R   A   L   K   K   N   T   N   S   E   K   A   N   I   Q   A   K   N   D
1981                                                2011
GAA ACC TAT CTG TTT CTT CAG GAT CAG GAT GAA AGC GCT GAT TCG CAT CAC CAT GAC GAG
E   T   Y   L   F   L   Q   D   Q   D   E   S   A   D   S   H   H   H   D   E
2041                                                2071
TTA GGT TCA GAA ATC ACT TTG GCT GAC AAT AAG TTT TCT TAT TTG CCC CCA ACT CTA GAA
L   G   S   E   I   T   L   A   D   N   K   F   S   Y   L   P   P   T   L   E
2101                                                2131
GAG TTG ATG GAA GAG CAG GAC TGT AAC AAT GGC AGA TCT TTT AAA AAT TTC ATG TTT TCC
E   L   M   E   E   Q   D   C   N   N   G   R   S   F   K   N   F   M   F   S
2161                                                2191
AAC GAT ACC GGT ATT GAC GGT AGT GCC GGT ACT GAT GAC GAC TAC ACC AAA GTT CTG AAA
N   D   T   G   I   D   G   S   A   G   T   D   D   D   Y   T   K   V   L   K
2221                                                2251
```

```
TCC AAA AAA ATT TCT ACG TCG AAG TCG AAC GCT AAC CTT TAT GAC TTA AAC GAT AAC AAC
S   K   K   I   S   T   S   K   S   N   A   N   L   Y   D   L   N   D   N   N
2281                                                2311
AAT GAT GCA ACT GCC ACC AAT GAA CTT GAT CAA AGC AGT TTC ATC GAC GAC CTT GAC GAA
N   D   A   T   A   T   N   E   L   D   Q   S   S   F   I   D   D   L   D   E
2341                                                2371
GAT GTC GAT TTT TTA Aag gta caa gta ttt tga aat agg cat gtt gca ata aaa cga aaa
D   V   D   F   L   K   V   Q   V   F   *

2401                                                2431
caa cta aaa atc acg aaa aca aaa tga tat tat aca ata aaa aat tct tat tat ggg taa

2461                                                2491
tga tag tat tct tcg cct gct tag gcg tcc ttt tcc ttc aac aac aaa aat tcc aaa aaa

2521                                                2551
aaa aag taa aaa aac aaa act ttg att gtt ttt taa tga tgt taa tga ttt ttt ttt tct

2581                                                261
ttc ttt atc ata aaa aaa aag tta aaa tga aaa aca aat atg ggt ctg gaa ggc cat tat

2641                                                2671
ttt ttt ttt att tat ata ccg ttt ctg gta ctt agt tat tta ttc tca tac ata cac tat

2701                                                2731
att caa att acc taa gag cat ttt cac ata tcc gtt tac ttt cat ttt ttt ttt ttt tgc

2761                                                2791
ttc ctt ttt aca tat ctt ccg tat atc aca tca cgt tta cgc gta tgg tga aac acg tca

2821                                                2851
aga gaa aaa tga taa aat caa att ttg att tac atc agg ctc cac agg aca ggg aaa tct

2881                                                2911
atc tag tga ggc gat aac tgt agt tcg atg tac tca ttt gaa ctg gac aaa ttg aaa att

2941                                                2971
gag ctg aaa aca tgg gag cat gat ttc att gat aaa aat aaa agg gaa ccc aca agg gat

3001                                                3031
gac atc aag agc ctg cgg act gtt cgg cag atg tat aaa caa tat tcc aca ctg aag aag

3061                                                3091
aaa caa tct ttg caa cga caa aaa gtt gac act caa gag tcg gtt gaa ctc ccg gca cat

3121                                                3151
aaa aaa gac cac gac gaa gtc gta gag ata ggc cct act ccc caa gtt tac ggt aag gcg

3181                                                3211
att agt atc ttt gac atg aat ttg tcg cct ata aag cct ata tac atg aca ttc aca aat

3241                                                3271
aat att gat gtt aac aat gat aac tcc aag aca att tct aat gaa tct tct cca cga aaa

3301                                                3331
act att ctc cta aaa tcg tcg cct gca gga att cga tat caa gct tat cga tac cgt cga
```

Figure 12B. Nucleotide and amino acid sequence of the 402 bp TEF2 promoter fused to the HAP4 coding region, as cloned in pKSPO2 and integrated in the yeast genome

```
2                                                  32
agc tca atg ttt cta ctc ctt ttt tac tct tcc aga ttt tct cgg act ccg cgc atc gcc

62                                                 92
gta cca ctt caa aac acc caa gca cag cat act aaa ttt ccc ctc ttt ctt cct cta ggg

122                                                152
tgt cgt taa tta ccc gta cta aag gtt tgg aaa aga aaa aag aga ccg cct cgt ttc ttt

182                                                212
ttc ttc gtc gaa aaa ggc aat aaa aat ttt tat cac gtt tct ttt tct tga aaa ttt ttt

242                                                272
ttt ttg att ttt ttc tct ttc gat gac ctc cca ttg ata ttt aag tta ata aac ggt ctt

302                                                332
caa ttt ctc aag ttt cag ttt cat ttt tct tgt tct att aca act ttt ttt act tct tgc

362                                                392
tca tta gaa aga aag cat agc aat cta atc taa gtt tta att aca aat cta gaa cta gtg

422                                                452
gat ccc ccc ATG ACC GCA AAG ACT TTT CTA CTA CAG GCC TCC GCT AGT CGC CCT CGT AGT
          M   T   A   K   T   F   L   L   Q   A   S   A   S   R   P   R   S
482                                                512
AAC CAT TTT AAA AAT GAG CAT AAT AAT ATT CCA TTG GCG CCT GTA CCG ATC GCC CCA AAT
N   H   F   K   N   E   H   N   N   I   P   L   A   P   V   P   I   A   P   N
542                                                572
ACC AAC CAT CAT AAC AAT AGT TCG CTG GAA TTC GAA AAC GAT GGC AGT AAA AAG AAG AAG
T   N   H   H   N   N   S   S   L   E   F   E   N   D   G   S   K   K   K   K
602                                                632
AAG TCT AGC TTG GTG GTT AGA ACT TCA AAA CAT TGG GTT TTG CCC CCA AGA CCA AGA CCT
K   S   S   L   V   V   R   T   S   K   H   W   V   L   P   P   R   P   R   P
662                                                692
GGT AGA AGA TCA TCT TCT CAC AAC ACT CTA CCT GCC AAC AAC ACC AAT AAT ATT TTA AAT
G   R   R   S   S   S   H   N   T   L   P   A   N   N   T   N   N   I   L   N
722                                                752
GTT GGC CCT AAC AGC AGG AAC AGT AGT AAT AAT AAT AAT AAT AAT AAC ATC ATT TCG AAT
V   G   P   N   S   R   N   S   S   N   N   N   N   N   N   I   I   S   N
782                                                812
AGG AAA CAA GCT TCC AAA GAA AAG AGG AAA ATA CCA AGA CAT ATC CAG ACA ATC GAT GAA
R   K   Q   A   S   K   E   K   R   K   I   P   R   H   I   Q   T   I   D   E
842                                                872
AAG CTA ATA AAC GAC TCG AAT TAC CTC GCA TTT TTG AAG TTC GAT GAC TTG GAA AAT GAA
K   L   I   N   D   S   N   Y   L   A   F   L   K   F   D   D   L   E   N   E
902                                                932
AAG TTT CAT TCT TCT GCC TCC TCC ATT TCA TCT CCA TCT TAT TCA TCT CCA TCT TTT TCA
K   F   H   S   S   A   S   S   I   S   S   P   S   Y   S   S   P   S   F   S
962                                                992
AGT TAT AGA AAT AGA AAA AAA TCA GAA TTC ATG GAC GAT GAA AGC TGC ACC GAT GTG GAA
S   Y   R   N   R   K   K   S   E   F   M   D   D   E   S   C   T   D   V   E
```

```
1022                                                    1052
ACC ATT GCT GCT CAC AAC AGT CTG CTA ACA AAA AAC CAT CAT ATA GAT TCT TCT TCA AAT
T   I   A   A   H   N   S   L   L   T   K   N   H   H   I   D   S   S   S   N
1082                                                    1112
GTT CAC GCA CCA CCC ACG AAA AAA TCA AAG TTG AAC GAC TTT GAT TTA TTG TCC TTA TCT
V   H   A   P   P   T   K   K   S   K   L   N   D   F   D   L   L   S   L   S
1142                                                    1172
TCC ACA TCT TCA TCG GCC ACT CCG GTC CCA CAG TTG ACA AAA GAT TTG AAC ATG AAC CTA
S   T   S   S   S   A   T   P   V   P   Q   L   T   K   D   L   N   M   N   L
1202/401                                                1232
AAT TTT CAT AAG ATC CCT CAT AAG GCT TCA TTC CCT GAT TCT CCA GCA GAT TTC TCT CCA
N   F   H   K   I   P   H   K   A   S   F   P   D   S   P   A   D   F   S   P
1262                                                    1292
GCA GAT TCA GTC TCG TTG ATT AGA AAC CAC TCC TTG CCT ACT AAT TTG CAA GTT AAG GAC
A   D   S   V   S   L   I   R   N   H   S   L   P   T   N   L   Q   V   K   D
1322                                                    1352
AAA ATT GAG GAT TTG AAC GAG ATT AAA TTC TTT AAC GAT TTC GAG AAA CTT GAG TTT TTC
K   I   E   D   L   N   E   I   K   F   F   N   D   F   E   K   L   E   F   F
1382                                                    1412
AAT AAG TAT GCC AAA GTC AAC ACG AAT AAC GAC GTT AAC GAA AAT AAT GAT CTC TGG AAT
N   K   Y   A   K   V   N   T   N   N   D   V   N   E   N   N   D   L   W   N
1442                                                    1472
TCT TAC TTA CAG TCT ATG GAC GAT ACA ACA GGT AAG AAC AGT GGC AAT TAC CAA CAA GTG
S   Y   L   Q   S   M   D   D   T   T   G   K   N   S   G   N   Y   Q   Q   V
1502                                                    1532
GAC AAT GAC GAT AAT ATG TCT TTA TTG AAT CTG CCA ATT TTG GAG GAA ACC GTA TCT TCA
D   N   D   D   N   M   S   L   L   N   L   P   I   L   E   E   T   V   S   S
1562                                                    1592
GGG CAA GAT GAT AAG GTT GAG CCA GAT GAA GAA GAC ATT TGG AAT TAT TTA CCA AGT TCA
G   Q   D   D   K   V   E   P   D   E   E   D   I   W   N   Y   L   P   S   S
1622                                                    1652
AGT TCA CAA CAA GAA GAT TCA TCA CGT GCT TTG AAA AAA AAT ACT AAT TCT GAG AAG GCG
S   S   Q   Q   E   D   S   S   R   A   L   K   K   N   T   N   S   E   K   A
1682                                                    1712
AAC ATC CAA GCA AAG AAC GAT GAA ACC TAT CTG TTT CTT CAG GAT CAG GAT GAA AGC GCT
N   I   Q   A   K   N   D   E   T   Y   L   F   L   Q   D   Q   D   E   S   A
1742/581                                                1772
GAT TCG CAT CAC CAT GAC GAG TTA GGT TCA GAA ATC ACT TTG GCT GAC AAT AAG TTT TCT
D   S   H   H   H   D   E   L   G   S   E   I   T   L   A   D   N   K   F   S
1802                                                    1832/611
TAT TTG CCC CCA ACT CTA GAA GAG TTG ATG GAA GAG CAG GAC TGT AAC AAT GGC AGA TCT
Y   L   P   P   T   L   E   E   L   M   E   E   Q   D   C   N   N   G   R   S
1862                                                    1892
TTT AAA AAT TTC ATG TTT TCC AAC GAT ACC GGT ATT GAC GGT AGT GCC GGT ACT GAT GAC
F   K   N   F   M   F   S   N   D   T   G   I   D   G   S   A   G   T   D   D
1922                                                    1952
GAC TAC ACC AAA GTT CTG AAA TCC AAA AAA ATT TCT ACG TCG AAG TCG AAC GCT AAC CTT
D   Y   T   K   V   L   K   S   K   K   I   S   T   S   K   S   N   A   N   L
1982                                                    2012
TAT GAC TTA AAC GAT AAC AAC AAT GAT GCA ACT GCC ACC AAT GAA CTT GAT CAA AGC AGT
Y   D   L   N   D   N   N   N   D   A   T   A   T   N   E   L   D   Q   S   S
2042                                                    2072/691
TTC ATC GAC GAC CTT GAC GAA GAT GTC GAT TTT TTA Aag gta caa gta ttt tga aat agg
F   I   D   D   L   D   E   D   V   D   F   L   K   V   Q   V   F   *
2102                                                    2132
cat gtt gca ata aaa cga aaa caa cta aaa atc acg aaa aca aaa tga tat tat aca ata

2162                                                    2192
aaa aat tct tat tat ggg taa tga tag tat tct tcg cct gct tag gcg tcc ttt tcc ttc
```

2222 2252
aac aac aaa aat tcc aaa aaa aaa aag taa aaa aac aaa act ttg att gtt ttt taa tga

2282 2312
tgt taa tga ttt ttt ttt tct ttc ttt atc ata aaa aaa aag tta aaa tga aaa aca aat

2342 2372
atg ggt ctg gaa ggc cat tat ttt ttt ttt att tat ata ccg ttt ctg gta ctt agt tat

2402 2432
tta ttc tca tac ata cac tat att caa att acc taa gag cat ttt cac ata tcc gtt tac

2462 2492
ttt cat ttt ttt ttt ttt tgc ttc ctt ttt aca tat ctt ccg tat atc aca tca cgt tta

2522 2552
cgc gta tgg tga aac acg tca aga gaa aaa tga taa aat caa att ttg att tac atc agg

2582 2612
ctc cac agg aca ggg aaa tct atc tag tga ggc gat aac tgt agt tcg atg tac tca ttt

2642 2672
gaa ctg gac aaa ttg aaa att gag ctg aaa aca tgg gag cat gat ttc att gat aaa aat

2702 2732
aaa agg gaa ccc aca agg gat gac atc aag agc ctg cgg act gtt cgg cag atg tat aaa

2762 2792/931
caa tat tcc aca ctg aag aag aaa caa tct ttg caa cga caa aaa gtt gac act caa gag

2822 2852
tcg gtt gaa ctc ccg gca cat aaa aaa gac cac gac gaa gtc gta gag ata ggc cct act

2882 2912
ccc caa gtt tac ggt aag gcg att agt atc ttt gac atg aat ttg tcg cct ata aag cct

2942 2972
ata tac atg aca ttc aca aat aat att gat gtt aac aat gat aac tcc aag aca att tct

3002 3032
aat gaa tct tct cca cga aaa act att ctc cta aaa tcg tcg cct gca gga att cga tat

3062
caa gct tat cga tac cgt cga

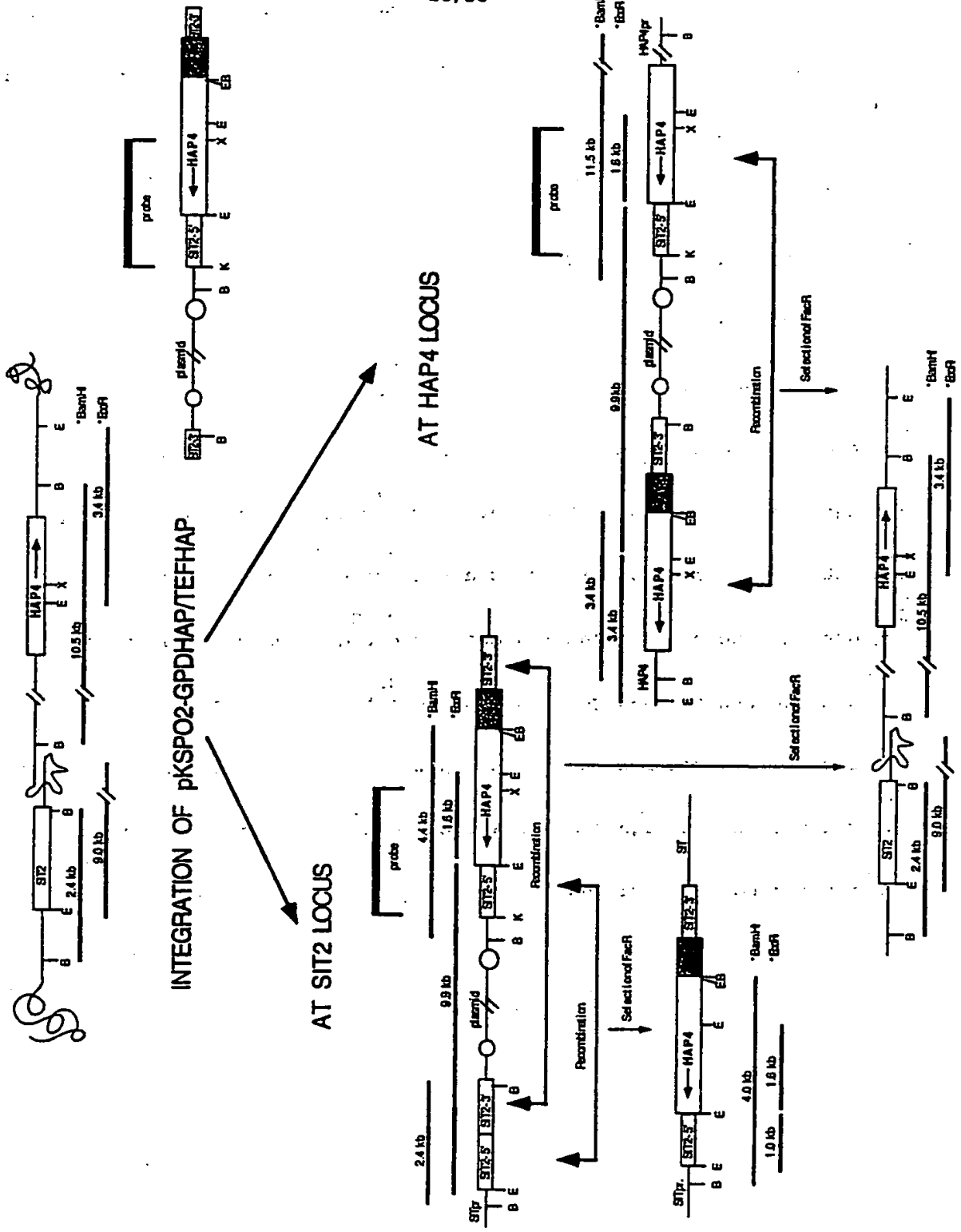
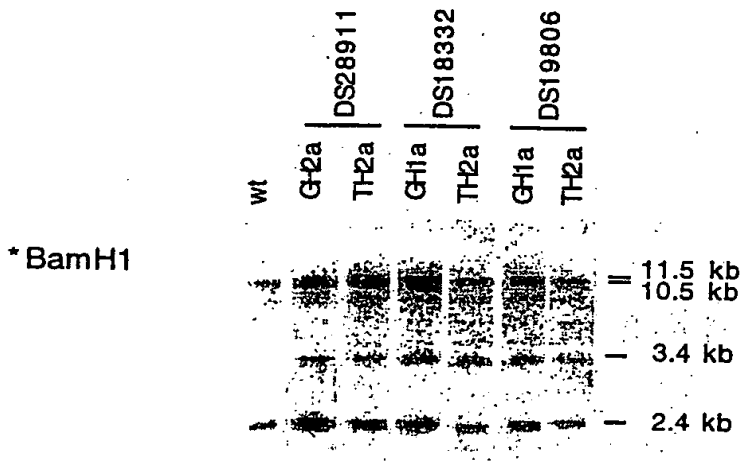

INTEGRATION OF pKSPO2-GPDHAP/TEFHAP
AT SIT2 LOCUS
AT HAP4 LOCUS
probe
plasmid
HAP4
SIT2-3'
SIT2
Recombination
Selection/FacR
BamH
EcoR
9.9 kb
3.4 kb
10.5 kb
9.0 kb
2.4 kb
1.8 kb
4.4 kb
11.5 kb
4.0 kb
1.0 kb

Figure 13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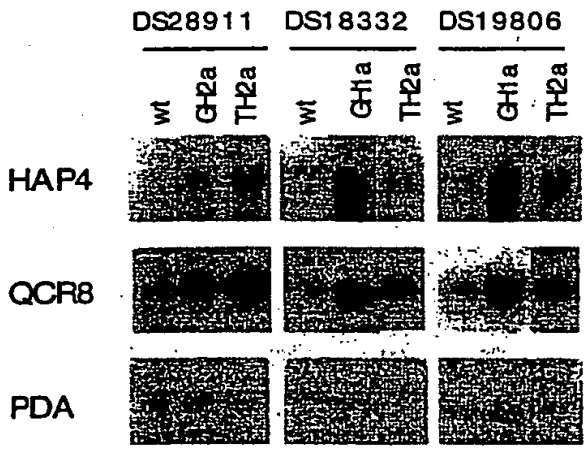
TH2a
DS28911
GH1a
TH2a
DS18332
GH1a
TH2a
DS19806
*BamH1
11.5 kb
10.5 kb
3.4 kb
2.4 kb

Figure 14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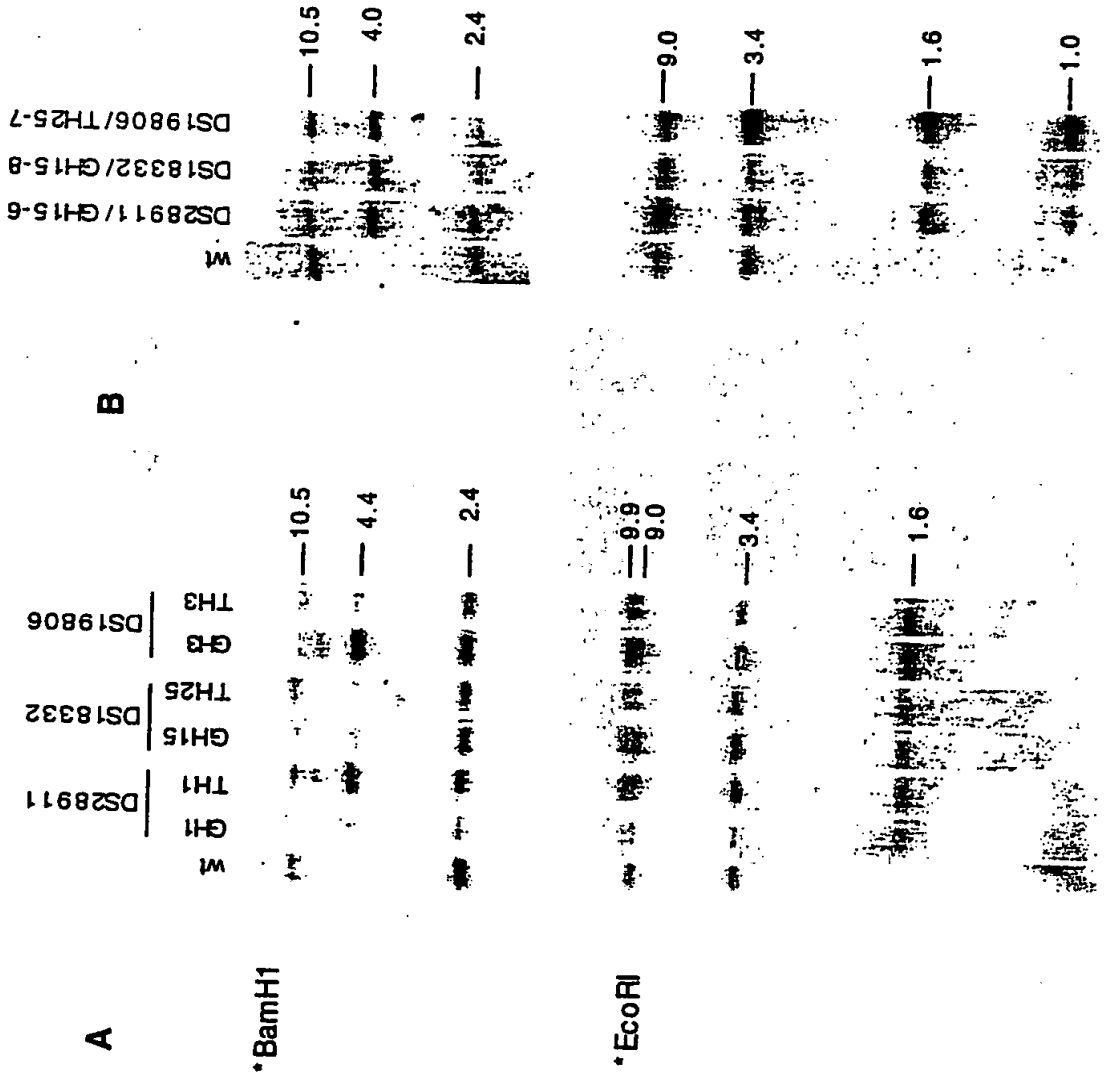
DS18332
DS19806
wt
GH2a
TH2a
wt
GH1a
TH2a
wt
GH1a
TH2a
HAP4
QCR8
PDA

Figure 15


A
*BamH1
*EcoRI
wt
GH1
TH1
DS289 11
GH15
TH25
DS18332
GH3
TH3
DS19806
10.5
4.4
2.4
9.9
9.0
3.4
1.6
B
wt
DS289 11/GH15-6
DS18332/GH15-8
DS19806/TH25-7
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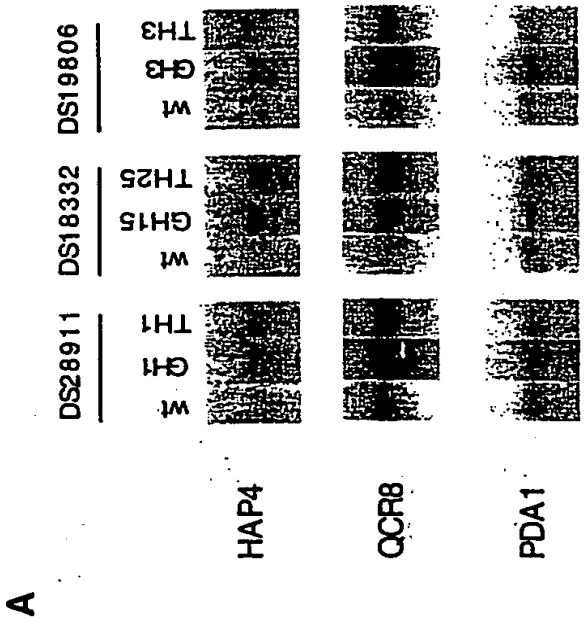
4.0
2.4
9.0
3.4
1.6
1.0

Figure 16


A
HAP4
QCR8
PDA1
DS28911
wt
GH1
TH1
DS18332
wt
GH15
TH25
DS19806
wt
GH3
TH3

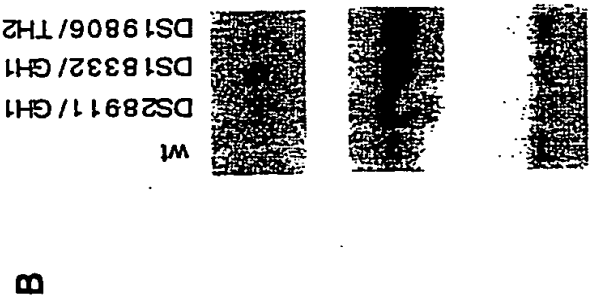

B
wt
DS28911/GH15-6
DS18332/GH15-8
DS19806/TH25-7

Figure 17

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 97/00688

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/81 C07K14/395 C12N1/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MULDER, WIETSE ET AL: "Distinct transcriptional regulation of a gene coding for a mitochondrial protein in the yeasts Saccharomyces cerevisiae and Kluyveromyces lactis despite similar promoter structures" MOL. MICROBIOL. (1995), 17(5), 813-24 CODEN: MOMIEE;ISSN: 0950-382X, 1995, XP000671075	1-14, 16-24
Y	see the whole document	15
	-/--	

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 May 1998	20/05/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hix, R

INTERNATIONAL SEARCH REPORT

Inte Jonal Application No
PCT/NL 97/00688

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	N. BONNEFOY ET AL.: "Yeast proteins can activate expression through regulatory sequences of the amdS gene of Aspergillus nidulans." MOL GEN GENET, vol. 246, 1995, pages 223-227, XP000670345	19-24
Y	see the whole document	1-18
X	J. DE WINDE ET AL.: "Regulation of mitochondrial biogenesis in Saccharomyces cerevisiae in the promoter of the QCR8 gene" EUR. J. BIOCHEM., vol. 233, 1995, pages 200-208, XP000671195	19-24
Y	see the whole document	1-18
X	BOWMAN S B ET AL: "POSITIVE REGULATION OF THE LPD1 GENE OF SACCHAROMYCES -CEREVISIAE BY THE HAP2-HAP3- HAP4 ACTIVATION SYSTEM." MOL GEN GENET 231 (2). 1992. 296-303. CODEN: MGGEAE ISSN: 0026-8925, XP000671042	19-24
Y	see the whole document	1-18
X	ROSENKRANTZ M ET AL: "The HAP2,3,4 transcriptional activator is required for derepression of the yeast citrate synthase gene, CIT1." MOLECULAR MICROBIOLOGY 13 (1). 1994. 119-131. ISSN: 0950-382X, XP000671072	19-24
Y	see the whole document	1-18
Y	FORSBURG S L ET AL: "IDENTIFICATION AND CHARACTERIZATION OF HAP4 A THIRD COMPONENT OF THE CCAAT-BOUND HAP2-HAP3 HETEROMER." GENES DEV 3 (8). 1989. 1166-1178. CODEN: GEDEEP ISSN: 0890-9369, XP000671039 cited in the application see the whole document	15
A	COSCHIGANO P W ET AL: "PHYSIOLOGICAL AND GENETIC ANALYSIS OF THE CARBON REGULATION OF THE NAD-DEPENDENT GLUTAMATE DEHYDROGENASE OF SACCHAROMYCES -CEREVISIAE." MOL CELL BIOL 11 (9). 1991. 4455-4465. CODEN: MCEBD4 ISSN: 0270-7306, XP000671043 see the whole document	

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INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/NL 97/00688

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DANG V-D ET AL: "Cloning of the ASN1 and ASN2 genes encoding asparagine synthetases in Saccharomyces cerevisiae: Differential regulation by the CCAAT-box-binding factor." MOLECULAR MICROBIOLOGY 22 (4). 1996. 681-692. ISSN: 0950-382X, XP000671076 see the whole document	